Molecular Analysis of the Orthologous Wheat and Rye Powdery Mildew Resistance Genes *Pm3* and *Pm8*

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Summary

Plants are constantly attacked by potentially pathogenic organisms which can lead to severe disease outbreaks and yield losses in agricultural production. An effective, economical and environmentally sustainable approach is the deployment of resistance genes in elite crop cultivars. In wheat, more than 43 genetic loci have been described to mediate resistance against wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*), a potentially devastating fungal disease. The first powdery mildew resistance gene from wheat (*Pm3b*) was cloned in 2004. Subsequently, additional *Pm3* alleles were cloned from hexaploid and tetraploid wheat resulting in a total of 17 functional alleles known to date. The *Pm3* alleles belong to the large class of resistance genes encoding proteins with nucleotide-binding site (NB) and leucine-rich repeat (LRR) domains.

In a first project, we explored the race-specificity of *Pm3* alleles in comparison with the sequence diversity found among the alleles. The unique ARC2 domain of the proteins PM3A and PM3B was found to enhance effector-dependent resistance. This domain was able to broaden the resistance spectrum of the *Pm3f* allele in domain swap experiments in a single-cell transient expression assay. Therefore, this domain is contributing to the broad resistance spectrum observed for the *Pm3a* and *Pm3b* alleles. Furthermore, race-specificity of *Pm3* alleles was found to be mainly determined by the C-terminal LRRs. Finally, a *Pm3* allele with a broad resistance spectrum was designed by combining polymorphisms that determine the race specificity of *Pm3d* and *Pm3e* alleles.

In a second project, we wanted to clone a *Pm3* orthologous gene from rye which we hypothesized to be identical to the powdery mildew resistance gene *Pm8* present on the wheat-rye translocation chromosome 1BL.1RS in wheat. By homology-based cloning we molecularly isolated a candidate gene and showed that it mediates *Pm8*

race-specific powdery mildew resistance in a transient single-cell expression assay and in stably transformed wheat lines. A specific PCR marker for the candidate gene mapped to the previously described *Pm8* locus in two different mapping populations, further confirming the identity of the cloned gene. Sequence analysis of *Pm3* and *Pm8* revealed their orthologous relationship and suggested that they recognize similar effector molecules.

In a third project, we aimed at elucidating the molecular mechanism at the basis of suppression of *Pm8*-mediated powdery mildew resistance in some wheat cultivars. First, we could show that such lines contain an intact and expressed *Pm8* gene. Since a suppressor gene for *Pm8* in an earlier study was mapped to the *Pm3* locus in wheat, we tested this gene for its ability to suppress *Pm8*-mediated resistance. Indeed, the *Pm3CS* allele suppressed *Pm8*-mediated powdery mildew resistance in wheat lines containing *Pm8* in a transient single-cell expression assay. This result was further confirmed in transgenic lines with combined *Pm8* and *Pm3* transgenes. Quantitative expression analysis as well as protein analysis revealed that suppression takes place at the post-translational level most likely involving protein-protein interaction between PM8 and PM3. These data provide a first molecular explanation for the suppression phenomenon frequently observed during wheat breeding and suggests ways to circumvent it in the future.

Zusammenfassung

Pflanzen werden fortwährend von potenziell krankmachenden Organismen angegriffen was zu schweren Krankheitsausbrüchen und dadurch zu hohen Ertragseinbussen in der Landwirtschaft führen kann. Eine effektive, wirtschaftliche und ökologisch nachhaltige Methode dagegen ist die Verwendung von Resistenzgenen in führenden Getreidesorten. In Weizen wurden mehr als 43 Genorte beschrieben, welche Resistenz gegen Weizenmehltau (*Blumeria graminis* f.sp. *tritici*), eine potenziell verheerende Pilzkrankheit, vermitteln können. *Pm3b* wurde 2004 als erstes Weizenmehltauresistenzgen kloniert. Seither wurden insgesamt 17 funktionelle *Pm3*-Allele aus hexaploidem und tetraploidem Weizen isoliert. Die *Pm3*-Allelele gehören zur Gruppe der am häufigsten vorkommenden Resistenzgene, welche für Proteine mit einer Nukleotidbindestelle- (NB) und Leucin-reicher-Repeat (LRR)-Domäne kodieren.

In einem ersten Projekt untersuchten wir die Rassenspezifizität der *Pm3*-Allele im Zusammenhang mit ihrer Sequenzdiversität. Die einzigartige ARC2-Domäne der PM3A- und PM3B-Proteine konnte die effektorabhängige Resistenz verstärken. So zeigte zum Beispiel ein chimäres PM3F-Protein, welches diese Domäne enthielt, ein erweitertes Resistenzspektrum in einem transienten Expressionsexperiment. Daher ist diese Domäne mitverantwortlich für das erweiterte Resistenzspektrum, welches für die *Pm3a*- und *Pm3b*-Allele beobachtet wurde. Desweiteren wurde gefunden, dass die Rassenspezifizität der *Pm3*-Allele hauptsächlich durch die C-terminalen LRRs bestimmt wird. Schlussendlich konnte ein *Pm3*-Allel mit einem erweiterten Resistenzspektrum kreiert werden, indem man die Polymorphismen, welche die Rassenspezifizität von *Pm3d* und *Pm3e* ausmachen, kombinierte.

Das Ziel eines zweiten Projekts war die Klonierung eines *Pm3*-orthologen Gens aus Roggen. Dieses Gen könnte identisch mit dem Mehltauresistenzgen *Pm8* sein,

welches in Weizen auf dem Weizen-Roggen-Translokationschromosom 1BL.1RS liegt. Homologiebasierend isolierten wir ein Kandidatengen molekular und zeigten sowohl in einem transienten Einzelzellen-Transformationsexperiment, als auch in stabil transformierten Weizenlinien, dass es *Pm8*-rassenspezifische Mehltauresistenz vermittelt. Ein spezifischer PCR Marker für das Kandidatengen wurde auf dem früher beschriebenen *Pm8*-Genort in zwei verschiedenen Populationen kartiert und bestätigt damit die Identität des klonierten Gens. Sequenzanalysen von *Pm3* und *Pm8* offenbarten deren orthologe Beziehung und legten die Erkennung von ähnlichen Effektormolekülen nahe.

Das Ziel eines dritten Projekts war die Aufklärung des molekularen Mechanismus, welcher für die Suppression der *Pm8*-vermittelten Mehltauresistenz in einigen Weizensorten verantwortlich ist. Zuerst konnten wir zeigen, dass supprimierte Linien ein intaktes und exprimiertes *Pm8*-Gen enthalten. Da ein Suppressorgen für *Pm8* in einer früheren Studie am Weizen *Pm3*-Genort kartiert worden war, testeten wir dieses Gen auf seine Fähigkeit, die *Pm8*-vermittelte Resistenz zu unterdrücken. In einem transienten Einzelzellen-Transformationsexperiment unterdrückte das *Pm3CS*-Allel tatsächlich die *Pm8*-vermittelte Mehltauresistenz in Weizenlinien, welche *Pm8* enthielten. Dieses Resultat konnte mit Hilfe von transgenen Linien, welche die *Pm8*- und *Pm3*-Transgene kombiniert enthielten, bestätigt werden. Quantitative Expressionsanalysen und Proteinanalysen zeigten, dass die Suppression nach der Translation stattfindet und mit grösster Wahrscheinlichkeit auf Protein-Protein-Interaktionen zwischen PM8 und PM3 basiert. Diese Daten liefern eine erste molekulare Erklärung für ein Suppressionsphänomen, welches regelmässig in der Weizenzüchtung beobachtet wird. Daraus können Möglichkeiten zur Verhinderung der Suppression in zukünftigen Züchtungsarbeiten abgeleitet werden.

1. General Introduction

1.1 The staple crops wheat and rye

1.1.1 Domestication and evolution

Wheat and rye belong to the grass family (Poaceae), which also includes some of the other most important crop species for human nutrition and animal feed such as maize and rice. Wheat (*Triticum* spp.) and rye (*Secale cereale*) together with barley (*Hordeum vulgare*) belong to the tribe Triticeae. Their domestication occurred more than 10'000 years ago in the Middle East, in the so called Fertile Crescent, when people started to settle down and practice farming during the Neolithic revolution (Charmet 2011, Salamini *et al.* 2002). Hexaploid bread wheat has three homoeologous genomes (*Triticum aestivum* L., 2n=6x=42, AABBDD), a result of the hybridization of the allotetraploid progenitor *T. turgidum* (AABB) and the diploid D-genome progenitor *Aegilops tauschii* (Kilian *et al.* 2007). In contrast to self-fertile hexaploid wheat, rye has a diploid genome (2n=2x=14, RR), is self-incompatible, and was domesticated after its wild progenitors had grown as weeds in wheat and barley fields of the near East as well as in Europe (Lundqvist 1956, Sencer and Hawkes 1980, Willcox 2005). Divergence of wild wheat and rye occurred 7 million years ago, long before their domestication by early farmers (Huang *et al.* 2002a, Huang *et al.* 2002b).

1.1.2 Trends in wheat production

Corn, wheat, and rice are the most important crop species for human food and animal feed supply in the world. In 2012 the world wheat production yielded 671 million tonnes, with China, India, and USA leading production (Food and Agriculture Organization of the United Nations, FAOSTAT, http://faostat.fao.org/). This is three times more than the wheat production in 1961 (222 million tonnes). This production increase is not the result of an enlargement of area under cultivation but is nearly solely

due to the production increase of wheat per acreage from 1.2 t/ha in 1961 to 3.4 t/ha in 2012 (FAOSTAT). This massive increase in wheat productivity in the last 50 years was achieved by growing higher yielding wheat varieties derived from dwarf plants, use of fertilizer, water irrigation and pest control with pesticides (Hedden 2003, Trewavas 2001). New agronomic techniques and crop protection measures have also increased yield by minimizing losses due to pests. Nevertheless, high yield losses are still caused by weeds, animal pests, and pathogens. Total actual wheat losses were estimated to be 29%, varying from 14% in Northwest Europe to over 35% in Central Africa. Highest losses are due to pathogens (10%), in regions with high productivity, and are most frequently caused by *Blumeria graminis*, *Septoria* spp. and rust fungi (Oerke 2006).

1.1.3 The complex genome of bread wheat

The members of the Triticeae tribe have very large genomes with a high amount of repetitive sequence. Therefore, their genome sequences have become only partially available recently and will only be finished in the next years (Bartoš *et al.* 2008, Brenchley *et al.* 2012, Martis *et al.* 2013, Mayer *et al.* 2012). Allohexaploid wheat has one of the largest Triticeae genomes (17 Gb) which consists of more than 80% repetitive sequence (Paux *et al.* 2008). This genome is five times larger than the human genome and several times the size of the rice (370-Mb) or maize (2.6 Gb) genomes. Rye is diploid but its genome has a size of ~8 Gb. It underwent substantial translocations during the evolution from an ancestral Triticeae progenitor and only chromosome 1 is completely collinear to wheat and barley chromosome 1 (Martis *et al.* 2013). In contrast to other crop species whose genomes were sequenced years ago, molecular genomic crop improvement based on sequences has only recently become feasible for the Triticeae tribe.

1.2 The pathogen powdery mildew

Powdery mildew is a fungal pathogen affecting a broad range of angiosperm plants, including many cultivated crop plants and other economically important species. Powdery mildew belongs to the ascomycete fungi of the order Erysiphales, comprising more than 800 species (Braun 2011). Powdery mildew of cereals is caused by the species *Blumeria graminis*, which is classified into eight *formae speciales* based on their strict host specialization. Wheat is infected by the powdery mildew *forma specialis* (f.sp.) *tritici* (*Bgt*) while rye is infected by f.sp. *secalis* (*Bgs*) (Wyand and Brown 2003). Powdery mildew is an obligate biotrophic parasite which depends on living host epidermal cells for its multiplication (Zhang *et al.* 2005). It mostly infects the upper leaf surface and the disease emerges early in the crop season when temperature and humidity conditions are favourable. Infection starts when an airborne conidiospore lands on the host leaf surface, adheres itself and begins germination by forming an appressorium. Beneath the appressorium a penetration peg forms, from where the fungus starts to penetrate the rigid host plant cell wall by physical and chemical forces mainly comprising turgor pressure and lytic enzymes (Pryce-Jones *et al.* 1999). A finger-like feeding structure, the so called haustorium, invaginates the host plasma membrane but remains separate from the host cell cytoplasm by the extrahaustorial membrane, a modified host plasma membrane formed upon invagination (Panstruga 2003). This intimate contact allows the fungus to obtain nutrients needed for its own metabolism from the host cell, and allows the fungus to deliver so called effector proteins into the host cell which suppress host defences and maintain the interaction (Micali *et al.* 2011, Panstruga 2003, Pliego *et al.* 2013, Weis *et al.* 2013). This allows the fungus to form secondary hyphae and haustoria in neighbouring cells and to asexually produce haploid spores in colonies, which due to their fluffy, powder-like structure gave powdery mildew its name. In dry, hot weather, conditions when host

leaves start to dry out, powdery mildew starts to reproduce sexually by forming fruiting bodies called chasmothecia, which release ascospores in the spring to start a new infection (Glawe 2008).

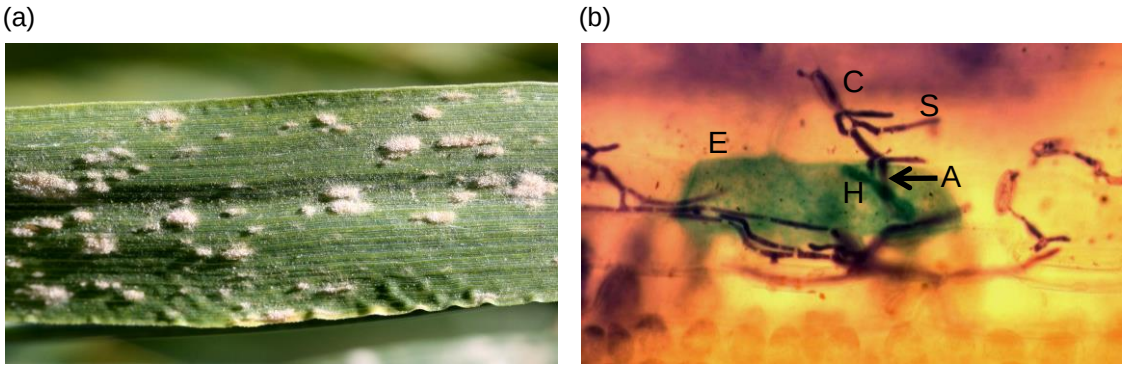

Figure 1. Macroscopic and microscopic view of wheat powdery mildew infection.
(a) Wheat powdery mildew colonies growing on the upper wheat leaf surface (picture taken by Susanne Brunner).
(b) Infection structures of wheat powdery mildew after successful penetration. When the conidiospore (C) lands on a host epidermal cell (E) it starts to germinate and form an appressorium (A). After penetration of the host cell wall it invaginates the host plasma membrane and forms a finger like feeding structure called a haustorium (H). Secondary hyphae (S) are then formed to infect neighbouring cells. The infected cell is stained in blue (GUS).

In earlier agricultural times, powdery mildew disease outbreaks were restricted to geographical regions with humid, rainfed climate. Due to irrigation of wheat fields, powdery mildew outbreaks are now also common in dryland areas and therefore occur nearly everywhere where wheat is cultivated. Certain agricultural techniques such as high seeding rate, the use of high amounts of nitrogen fertilizer, and the semi-dwarf morphology further boost powdery mildew growth and severe disease outbreaks (Bennett 1984, Shaner and

Finney 1977, Tompkins *et al.* 1992). Powdery mildew on wheat can result in fewer kernels per head, smaller kernel size in general (Bowen *et al.* 1991, Everts *et al.* 2001), higher tiller numbers that do not produce a grain head (Everts and Leath 1992), and reduced plant vigour due to reduced photosynthesis and transpiration rate (Shtienberg 1992). Several studies showed that wheat yield losses due to powdery mildew can be as high as 45 % under certain environmental and agricultural conditions, but its precise estimate is rather difficult and often lacks appropriate control groups (Bowen *et al.* 1991, Conner *et al.* 2003).

The biotrophic lifestyle of powdery mildew restricts its growth habitat to its host and therefore allows no *in vitro* cultivation, complicating its study under laboratory conditions. No reproducible transformation protocols could be established so far and cryo-cultivation is difficult and might result in the loss of isolates (Spanu and Panstruga 2012). This makes studies with powdery mildew not only time consuming but also complicates molecular studies of its interaction with its host plant. Recently the barley and wheat powdery mildew genomes have been sequenced and compared (Spanu *et al.* 2010, Wicker *et al.* 2013). It was found that obligate biotrophic powdery mildews have reduced their secondary metabolism and lost whole gene classes that might not be necessary, which is characteristic of a biotrophic lifestyle (Wicker *et al.* 2013). The availability of the powdery mildew genome sequences makes research on this pathogen easier. It might also give further insights into their biotrophy, explain their strict host specificity, and reveal the effector molecules that are necessary for successful host invasion (Pliego *et al.* 2013).

1.3 Disease resistance in plants

1.3.1 The plant immune system

Plants are constantly attacked by a broad range of potentially pathogenic microorganisms such as fungi, oomycetes, bacteria or viruses. The wax cuticle, rigid cell walls and lenticels provide natural physical barriers for pathogen entry. In addition, chemical barriers such as exudates of phytoanticipins and plant defensins on the plant

cell wall provide a first barrier to pathogens by inhibiting their entry into the host cell and the establishment of infection. However, pathogens have evolved sophisticated mechanisms to overcome such barriers. In response, plants have evolved an innate immune system based on two layers to defend against pathogen attacks. First, they recognize common microbial molecules such as bacterial flagellins, lipopolysaccharides or elongation factors, and fungal chitin or heptaglucosides. Such molecules are common among large classes of microbes and also represent common patterns of cell damage (Han and Jung 2013). These molecules are commonly referred to as PAMPs (or MAMPs) for pathogen (or microbe)-associated molecular patterns (Jones and Takemoto 2004, Marone *et al.* 2013) and are perceived by plant receptor proteins called pattern recognition receptors (PRRs). This recognition leads to general plant defence responses referred to as PAMP-triggered immunity (PTI) (Boyd *et al.* 2013, Dodds and Rathjen 2010). Pathogens evolved so called effector proteins which are delivered into host cells to suppress PTI and promote pathogen virulence by targeting plant defence pathways and metabolism (Boyd *et al.* 2013). Subsequently, plants evolved a second layer of recognition provided by intracellular disease resistance genes (*R* genes) to detect effector proteins and lead to effector triggered immunity (ETI) (Jones and Dangl 2006). The recognition of such effector/avirulence factors (AVRs) by R proteins induces defence responses associated with cell wall strengthening, phytoalexin biosynthesis and localised cell death termed hypersensitive response (HR) (Dodds and Rathjen 2010, Meng and Zhang 2013).These processes are qualitatively similar to PTI, but differ in quantitative expression. Defence signalling is not only restricted to local pathogen infection sites but can also induce systemic acquired resistance (SAR) in distant plant tissues leading to enhanced resistance in a later pathogen attack (Spoel and Dong 2012).

1.3.2 The NB-LRR resistance genes

The interaction between R proteins and AVRs was first genetically described in the gene-for-gene model, where a specific plant protein (*R* gene) recognizes a molecule from the pathogen (AVR gene) (Flor 1971). A direct interaction of R and AVR proteins was found for the flax *L* resistance alleles (Dodds *et al.* 2006) and the rice blast resistance gene *Pi-ta* (Jia *et al.* 2000), amongst others. However, it was found that gene-for-gene interaction very frequently relies on indirect recognition, e.g. the R proteins Bs3, Cf-2, N, Prf, RPM1,RPS2 or RPS5. These proteins sense alterations of proteins (guardees) modified by effectors (Caplan *et al.* 2008, Dangl and Jones 2001, DeYoung and Innes 2006, Van der Hoorn and Kamoun 2008). Most *R* genes recognizing AVRs from pathogens belong to the class of NB-LRR (Nucleotide-binding site, Leucine-rich repeat) proteins, which are similar to NOD-LRR proteins that are involved in apoptotic and inflammatory responses in the innate immune system of mammals (Ausubel 2005, Jones and Takemoto 2004). Based on the N-terminal domain, two main subclasses exist, the CC-NB-LRRs and TIR-NB-LRRs, where only the former is found in grasses (Pan *et al.* 2000). More than 50 NB-LRR proteins have been cloned from model as well as crop plants until 2011 (Joshi and Nayak 2011), and many more were identified in recent years (Marone *et al.* 2013).

The different subdomains of this protein class fulfil different functional roles during AVR recognition and plant defence signalling. The coiled-coil (CC) or TIR domain is thought to transfer and activate downstream signalling components due to its ability to mediate HR on its own (Bernoux *et al.* 2011, Maekawa *et al.* 2011a, Marone *et al.* 2013), and its interaction with transcription factors as in the case of barley MLA (Shen *et al.* 2007). Furthermore, intramolecular interactions of CC and TIR domains with the NB and LRR domains were shown (Moffett *et al.* 2002, Rairdan *et al.* 2008), as well as intermolecular interactions with their guarded proteins or downstream signalling

molecules (Caplan *et al.* 2008, Chang *et al.* 2013, Maekawa *et al.* 2011b, Shen *et al.* 2007). Experimental data and analysis of the flax L6 TIR and the MLA10 CC crystal structures suggested homodimerization of these domains (Bernoux *et al.* 2011, Maekawa *et al.* 2011b, Mestre and Baulcombe 2006). The NB domain can be divided into three subdomains; NB, ARC1 and ARC2 based on their homology to human APAF-1 and *C. elegans* CED-4 (McHale *et al.* 2006, Van der Biezen and Jones 1998b). The NB-ARC domain is thought to function as a molecular switch which induces conformational changes after pathogen recognition by the LRR domain and ATP hydrolysis (Collier and Moffett 2009, Takken *et al.* 2006). There, the ATP bound state is the active form, while in the ADP bound state the R protein is inactive. R proteins also consist of a precise succession of several highly conserved motifs which, if mutated, can lead to loss or gain-of-function mutants, while a mutation in the highly conserved MHD motif of the ARC2 domain leads to autoactivation of the resistance protein (Dinesh-Kumar *et al.* 2000, Takken *et al.* 2006, van Ooijen *et al.* 2008b, Williams *et al.* 2011). The LRR domain is thought to be involved in protein-protein interaction and to be the major determinant of recognition specificity (Padmanabhan *et al.* 2009). It consists of 20-30 LRRs: tandem arrays of short motifs with the leucine rich consensus sequence LxxLxLxx(N/C/T)xL (Kobe and Kajava 2001, Matsushima *et al.* 2007). The solvent exposed residues (x) are highly variable and are under diversifying selection (Marone *et al.* 2013). Intramolecular interaction between the NB-ARC domain and the C-terminal LRR domain keeps the R protein in a closed conformation in the absence of pathogen effectors (Takken and Goverse 2012).

1.3.3 Disease (powdery mildew) resistance genes in crop breeding

Crop improvement has relied strongly on breeding cereals for resistance to biotrophic, fungal pathogens. Effective resistance was found to either depend on several genes (polygenic) or on a single gene (monogenic). While the former can result in durable, quantitative resistance manifested in reduced disease, resistance based on a single *R* gene is usually qualitative and mediates complete resistance to a certain pathogen (Marone *et al.* 2013, Poland *et al.* 2009). *R* genes are usually race-specific, meaning that they are only effective against some races of a certain pathogen, and follow the gene-for-gene concept first described by Flor (Flor 1971). Due to the high evolutionary potential of many pathogens and the strong selection for virulent pathogen mutants under agricultural conditions, such *R* genes are often only effective for few years when deployed in extensively grown elite wheat cultivars (Jones and Dangl 2006, McDonald and Linde 2002). However, due to their high resistance level and the relative ease with which they can be manipulated and applied in breeding programs, many *R* genes were introduced from cultivated or wild wheat and close relatives into elite wheat cultivars to mediate and maintain resistance (Baum *et al.* 1992, Tyrka and Chelkowski 2004).

The use of powdery mildew resistance (*Pm*) genes in breeding programs started in the 1930s (Hsam and Zeller 2002). Since that time, more than 43 genetic loci have been described in wheat that mediate powdery mildew resistance (Bennett 1984, McIntosh *et al.* 2012); however only the *Pm3* gene and a key member of the *Pm21* resistance locus have been molecularly cloned (Cao *et al.* 2011, Yahiaoui *et al.* 2004). The *Pm3* gene confers race-specific resistance to powdery mildew *Blumeria graminis* f.sp. *tritici* and is located on the short arm of wheat chromosome 1 (Huang and Röder 2004). The gene was found to encode a CC-NB-LRR protein and so far 17 functional and 37 susceptible alleles were cloned from hexaploid and tetraploid wheat (Bhullar *et al.* 2010, Yahiaoui *et al.* 2004). Very high sequence conservation of the alleles indicated a

recent evolutionary origin of this allelic series after wheat domestication (Yahiaoui *et al.* 2006). The susceptible allele *Pm3CS* identified first from the wheat cultivar Chinese Spring represents the consensus sequence of all so far known *Pm3* alleles (Bhullar *et al.* 2010, Yahiaoui *et al.* 2006).

1.4 Wild relatives as genetic resources for wheat breeding

1.4.1 Alien chromosome introgressions for disease resistance

Deployment of disease resistance genes in wheat breeding is an effective, economical and environmentally friendly approach to protect crop plants from pathogen attack and to reduce yield loss caused by pathogenic organisms. However, due to the rapid adaptation of the pathogen to overcome a resistance gene under agricultural conditions, there is a constant need for new resistance genes that can be introduced into elite wheat cultivars (McDonald and Linde 2002, Wulff *et al.* 2011). Since current crop production often relies on few high yielding varieties with reduced genetic diversity, such new *R* gene sources have to be derived from landraces, wheat with lower ploidy, and close relatives (Baum *et al.* 1992). To date, more than 30 *R* genes/alleles have been transferred from wild relatives to wheat, demonstrating the practicability of alien genetic material for resistance breeding. In total, 15 wild relatives from the tribe Triticeae served as powdery mildew resistance sources, comprising members of the genera *Aegilops*, *Haynaldia*, *Secale*, *Thinopyrum* and *Triticum* (McIntosh *et al.* 2012, Tyrka and Chelkowski 2004). Since such alien chromosomes often show linkage drag because they do not recombine with the wheat genome, they therefore carry undesired genes along with the benefical one. Sophisticated methods are necessary to introduce desired *R* genes into wheat and also to reduce the amount of transferred chromatin (Baum *et al.* 1992, Gill *et al.* 2011). These methods include irradiation, mutation in the wheat pairing suppressor locus *Ph1*, induced homoeologous

pairing and recombination, somaclonal variation in tissue culture, cell culture and chemical treatment (Baum *et al.* 1992, Lukaszewski 2000, Qi *et al.* 2007, Zhang *et al.* 2001).

1.4.2 Rye as a valuable source for *R* genes

Rye proved to be a valuable source for resistance genes against several diseases when segments of its genome were introgressed into wheat. Resistance genes against powdery mildew, leaf/stem/ and yellow rust, wheat curl mite, Hessian fly, Russian aphid, greenbug and cereal cyst nematode were transferred from rye to wheat (McIntosh *et al.* 2012, Tyrka and Chelkowski 2004). However, these resistance genes resulted only from a very limited number of rye introgressions in wheat, and a high number of *R* genes/alleles in the rye genome remain unexplored. Several *R* genes were genetically mapped in the rye genome at low resolution, but due to the lack of appropriate genetic resources, only few resistance genes have been phenotypicaly analyzed and mapped at high resolution (Bolibok-Bragoszewska *et al.* 2009, Geiger *et al.* 1988, Kast and Geiger 1982, Lind and Züchner 1984, Melz *et al.* 1992, Riley and Macer 1966, Schlegel and Melz 1996, Tyrka and Chelkowski 2004). To date, none of them has been cloned in rye. This is in contrast to wheat genetics, where near isogenic lines were produced over decades for the identification of disease resistance genes (Briggle 1969). Lack of such genetic material in rye resulted mainly from its self-incompatibility, which makes generating near isogenic lines difficult (Hackauf and Wehling 2005, Lundqvist 1956).

1.4.3 The 1R wheat-rye translocation and substitution lines

One of the widest deployed foreign genetic sources in wheat is the rye chromosome 1R, either present as a substitution for a wheat chromosome, mostly 1B, or as a 1BL.1RS or 1AL.1RS translocation, where the short arm of 1R replaces wheat

chromosome 1BS or 1AS, respectively (Baum and Appels 1991). Even though the 1RS chromosome introgression in wheat had some negative effects on grain processing quality (Graybosch 2001), it was a success in wheat breeding due to the presence of several disease resistance genes, its wide adaptation, and mostly to its high yield potential (Kim *et al.* 2004, Rabinovich 1998, Sharma *et al.* 2009). Therefore, it is nowadays present in several hundred wheat cultivars, and wheat lines carrying the 1RS chromosome are cultivated at large scale (Purnhauser *et al.* 2011, Rabinovich 1998, Villareal *et al.* 1998). All these wheat lines are derivatives of only four events of 1RS introgessions in wheat at different locations; one in the USA, one in Japan (Tsunewaki 1964), and two in Germany (Mettin *et al.* 1973, Rabinovich 1998, Schlegel and Korzun 1997). In the USA, the wheat 'Amigo' line carrying a 1AL.1RS translocation was produced and carries the 1RS chromosome arm derived from the rye cultivar 'Insave' (Rabinovich 1998, Schlegel and Korzun 1997). This chromosome carries the powdery mildew resistance gene *Pm17*, the greenbug resistance gene *Gb2*, the stem rust resistance gene $Sr1RS^{Amigo}$, and provides tolerance to wheat curl mite (Graybosch 2001, Heun *et al.* 1990, Lowry *et al.* 1984, Mater *et al.* 2004, Olson *et al.* 2010, Rabinovich 1995). In Japan, a 1BL.1RS translocation from a Triticale source was produced mediating resistance to wheat curl mite (Tsunewaki 1964). However, this cultivar ('Salmon') has not been widely used in wheat breeding programs (Graybosch 2001).

From Germany, two sources of 1BL.1RS translocations are known, one produced in Salzmünde and one in Weihenstephan (Hsam and Zeller 2002). These cultivars are known as 'Salzmünder Bartweizen' and 'Zorba', and both are tracing back to the rye cultivar 'Petkus' (Schlegel and Korzun 1997, Szakács and Molnár-Láng 2008, Zeller 1973). The 1RS chromosome of this translocation carries the powdery mildew resistance gene *Pm8*, which mediates race-specific resistance to the wheat powdery

mildew (*Bgt*) pathogen (McIntosh 1988). However, soon after its widespread use in the 1970s, virulent powdery mildew isolates appeared, which had overcome the resistance (Bennett 1984, Heun and Friebe 1990). *Pm8* in wheat might be identical to the rye powdery mildew resistance gene *Pm1*, mapped on the 1RS rye chromosome arm (Börner and Korzun 1998, Korzun *et al.* 2001, Melz *et al.* 1992, Senft and Wricke 1996, Wricke *et al.* 1996). Besides the resistance gene *Pm8*, the translocation derived from Petkus carries three rust resistance genes: the leaf rust resistance gene *Lr26*, the stem rust resistance gene *Sr31*, and the yellow rust resistance gene *Yr9* (Singh *et al.* 1990). In a high resolution mapping population, these three genes were shown to map at different genetic loci but are actually located very close to each other and to *Pm8* (Mago *et al.* 2005, Mago *et al.* 2002).

It was speculated that the powdery mildew resistance genes *Pm17* and *Pm8*, located on the 1AL.1RS and 1BL.1RS translocation, respectively, could be allelic genes (Hsam and Zeller 1997). Therefore, the *Pm8* lines 'Helios' and 'Disponent' were crossed with the line Helami-105, in which the *Pm17* gene was transferred to a 1BL.1RS translocation, and the F_2 and F_3 progeny were analyzed with wheat powdery mildew isolates (Hsam *et al.* 1995, Hsam and Zeller 1997). Since no recombination was observed between *Pm17* and *Pm8*, they were suggested to be allelic. Mohler *et al*. (2001) developed an STS marker that distinguishes the two possible alleles. *Pm17* was also proposed to be allelic to the barley powdery mildew resistance gene *Mla* (Mohler *et al.* 2002).

1.4.4 Suppression of disease resistance genes

Resistance genes that are active in wild wheat species, or relatives with lower ploidy, often confer a lower level of resistance or do not mediate resistance at all after introduction into the hexaploid wheat background. The absence of resistance is usually

due to a suppressor gene present in the cultivar with the higher ploidy level (Hsam and Zeller 2002). This phenomenon, also frequently described to be caused by 'modifiers of resistance genes', has been observed several times during the production of synthetic hexaploid wheat, where usually a diploid wild grass (*Ae. tauschii*) is crossed with tetraploid wheat (Boyd 2005). Such suppression was observed for resistance to all three rust species (leaf, stem and yellow rust) in synthetic wheat. The suppressor genes appeared to be resistance-gene specific and could be located on any of the three parental wheat genomes (A, B or D) (Assefa and Fehrmann 2004, Kema *et al.* 1995, Ma *et al.* 1995). A specific suppressor gene (*SuLr23*) was identified for the leaf rust resistance gene *Lr23* on chromosome 2BS. This suppressor gene was mapped to the likely homoeologous loci on chromosome arm 2DS (McIntosh *et al.* 2011, Nelson *et al.* 1997). Similarly, suppression of the rye powdery mildew resistance gene *Pm8* in certain wheat lines was associated with the presence of a possible orthologous locus in the wheat genome (McIntosh *et al.* 2011). It was observed that not all translocation lines supposed to carry *Pm8* showed *Pm8* mediated powdery mildew resistance. In these lines, the presence of *Pm8* was only assumed based on the rust resistance of the translocation lines mediated by genes closely linked to *Pm8* (Friebe *et al.* 1989, Hanusova *et al.* 1996, Lutz *et al.* 1992). Zeller and Hsam (1996) located a dominant suppressor gene on wheat chromosome 7D. In contrast, Ren *et al*. (1996) found a suppressor gene on chromosome 1AS by association of suppression with the storage-protein locus on this chromosome. Since this gliadin storage gene is located in close proximity to the *Pm3* locus, McIntosh *et al*. (2011) hypothesised an involvement of this locus in suppression. Indeed, they found that suppression correlated with a marker derived from the 5' sequence of the *Pm3* gene. Suppression was not only found for *Pm8* and rust resistance genes, but also for resistance genes to other diseases and in crop species other than wheat. For example, suppression to tan spot disease was found in wheat (Siedler *et al.* 1994), to crown rust in oat (Wilson and McMullen 1997),

to soybean rust in soybean (Garcia *et al.* 2011) and to late blight in potato (Ordoñez *et al.* 1997), suggesting that suppression of *R* genes in plants is widespread.

1.5 Aim of this thesis

The aim of the first part of this thesis was to explore the sequence diversity between functional *Pm3* alleles and how they influence the race-specificity of *Pm3* alleles. In a follow up study it was tested if it is possible to broaden the race spectrum of *Pm3* by combining specificities of single *Pm3* alleles. The goal of the second part of the thesis was to clone, by homology-based cloning, candidate genes for the wheat powdery mildew resistance gene *Pm8*, a possible ortholog of *Pm3*, and to test such candidates for *Pm8*-mediated resistance function. In a further step, the molecular mechanism causing suppression of *Pm8*-mediated resistance in certain wheat cultivars was explored.

2. Intragenic allele pyramiding combines different specificities of wheat *Pm3* resistance alleles

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2.1 Summary

Some plant resistance genes occur as allelic series, with each member conferring specific resistance against a subset of pathogen races. In wheat, there are 17 alleles of the *Pm3* gene. They encode nucleotide-binding (NB-ARC) and leucine-rich-repeat (LRR) domain proteins, which mediate resistance to distinct race spectra of powdery mildew. It is not known if specificities from different alleles can be combined to create resistance genes with broader specificity. Here, we used an approach based on avirulence analysis of pathogen populations to characterize the molecular basis of *Pm3* recognition spectra. A large survey of mildew races for avirulence on the *Pm3* alleles revealed that *Pm3a* has a resistance spectrum that completely contains that of *Pm3f*, but also extends towards additional races. The same is true for the *Pm3b* and *Pm3c* gene pair. The molecular analysis of these allelic pairs revealed a role of the NB-ARC protein domain in the efficiency of effector-dependent resistance. Analysis of the wild-type and chimeric *Pm3* alleles identified single residues in the C-terminal LRR motifs as the main determinant of allele specificity. Variable residues of the N-terminal LRRs are necessary, but not sufficient, to confer resistance specificity. Based on these data, we constructed a chimeric *Pm3* gene by intragenic allele pyramiding of *Pm3d* and *Pm3e* that showed the combined resistance specificity and, thus, a broader recognition spectrum compared with the parental alleles. Our findings support a model of stepwise evolution of *Pm3* recognition specificities.

2.2 Introduction

Plants evolved two lines of defence against pathogen infections (Dodds and Rathjen 2010). The first is the basal resistance that relies on pre-formed physical and chemical barriers, and an immune system that induces defence responses upon detection of pathogen-associated molecular patterns (PAMPs; Schwessinger and Zipfel 2008). To fight microbes that produce effector molecules suppressing this resistance, plants evolved a second line of defence, which detects the presence or the action of pathogen effectors (Chisholm *et al.* 2006, Jones and Dangl 2006). This recognition is mediated by the products of resistance (*R*) genes, which induce a strong resistance reaction that stops the infection. Such a pathogen is avirulent on the host, and the detected effector an avirulence (Avr) factor. The pairwise interaction between *R* and *Avr* gene products is characterized genetically as gene-for-gene resistance (Flor 1971). Although R proteins recognize and respond to a wide variety of pathogen-derived effectors, they are built from a very limited set of modular domains (Dangl and Jones 2001). These include a nucleotide-binding (NB) domain followed by ARC1, ARC2 (thus NB-ARC) and a C-terminal leucine-rich-repeat (LRR) domain. The ARC subdomains were named after their presence in the human apoptotic protease-activating factor 1 (APAF-1), R proteins and the *Caenorhabditis elegans* Death-4 (CED-4) protein (van der Biezen and Jones 1998a). NB-ARC domains are highly similar in structure to mammalian NACHT domains (Albrecht and Takken 2006). In the plant R proteins I-2, Mi-1 and N, they were shown to bind and hydrolyse ATP (Tameling *et al.* 2002, Ueda *et al.* 2006). These findings, together with further structure-function analyses, indicate that the NB-ARC domain works as a reversible molecular switch (Danot *et al.* 2009, Lukasik and Takken 2009, Rairdan and Moffett 2007, Takken *et al.* 2006).

The LRR domain is a tandem array of repeats that are typically 20–29 amino acids long (Kobe and Kajava 2001). Each repeat contains a conserved motif with the consensus

sequence LxxLxLxxN(Cx)xL (Wei *et al.* 2008). Crystal structures of LRR domains revealed that the second conserved leucine and adjacent residues in the consensus sequence form a short β-strand, and that the β-strands of the different LRRs are arranged in parallel (Enkhbayar *et al.* 2004). These parallel β-strands form a β-sheet that lines the concave face of a horseshoe-shaped structure. The first five of the x-residues are exposed on the concave surface, and several studies demonstrated their involvement in the binding of interaction partners (reviewed in Bella *et al.* 2008). In plant R proteins, these residues are thought to mediate recognition specificity, as they were shown to be highly variable and under diversifying selection (e.g. Ellis *et al.* 2000a, Mondragon-Palomino *et al.* 2002, Seeholzer *et al.* 2010). Direct evidence for a role in specificity determination comes from domain swap and mutagenesis experiments (reviewed by DeYoung and Innes 2006, Dunning *et al.* 2007). However, there is emerging evidence that the LRR domain is also involved in diverse intra- and intermolecular interactions, which are not directly implicated in pathogen recognition, but contribute to R protein activity regulation (Jones and Takemoto 2004, Lukasik and Takken 2009, McDowell and Simon 2006).

Many *R* genes confer resistance only to a subset of all existing pathogen races. Among them is the multiallelic *Pm3* locus from hexaploid wheat (*Triticum aestivum* L.), which confers race-specific resistance to wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*). PM3 belongs to the subgroup of NB-LRR proteins encoding an N-terminal coiled-coiled (CC) domain. In the modern bread wheat gene pool, it occurs in seven, functionally distinct, true alleles, *Pm3a–Pm3g*, which have been molecularly isolated (Srichumpa *et al.* 2005, Yahiaoui *et al.* 2006, Yahiaoui *et al.* 2004). Recently, the alleles *Pm3k–Pm3t* were cloned from tetraploid wheat species and hexaploid wheat landraces (Bhullar *et al.* 2009, Bhullar *et al.* 2010, Yahiaoui *et al.* 2009). The *Pm3*

resistance alleles are highly similar in sequence to the susceptible allele *Pm3CS*, which also represents the consensus sequence of all resistance alleles (Yahiaoui *et al.* 2006).

To improve disease resistance of plants, it is advantageous to make use of *R* genes conferring broad spectrum resistance. The artificial extension of the recognition spectrum was successful in the Rx protein conferring viral resistance in potato (*Solanum tuberosum*) (Farnham and Baulcombe 2006). In flax (*Linum usitatissimum*), detailed molecular studies were performed on *R* alleles *L5*, *L6* and *L7*, which show overlapping resistance specificities to flax rust (*Melampsora lini*) (Dodds *et al.* 2006, Luck *et al.* 2000, Wang *et al.* 2007). Recombinant *L* genes were found to confer resistance to only a subset of the rust strain recognized by either of the parental *L* alleles (Ellis *et al.* 1999, Luck *et al.* 2000). Apart from the flax *L* alleles, relatively little is known about the resistance spectra and their overlap for different *R* alleles from a specific locus. Therefore, it is not clear if natural allelic specificities might be combined. Here, we tested a large set of powdery mildew isolates for recognition by *Pm3a–Pm3g* alleles to determine whether there are natural examples for extensions in recognition capacities. Indeed, two *Pm3* pairs were identified where one allele recognized all pathogen races that are also recognized by the second allele, but this one allele extended the recognition spectrum to an additional set of mildew isolates. To elucidate the molecular mechanism leading to these functional differences, a series of domain swap experiments was performed. The very low number of sequence polymorphisms between functionally different *Pm3* alleles makes them an ideal system to study the molecular basis of race specificity. Based on the results obtained, we investigated if it is possible to rationally design *Pm3* genes with broadened disease resistance by combining specificities from different *Pm3* alleles. We could demonstrate that intragenic allele pyramiding of *Pm3d* and *Pm3e* leads to a functional gene with dual resistance specificities, thus achieving an extended resistance spectrum.

2.3 Results

2.3.1 Two pairs of *Pm3* alleles with narrow and extended resistance spectra

We carried out a virulence survey for *Pm3a–Pm3g* alleles based on 102 isolates in 2007 to determine the avirulence spectra of wheat powdery mildew isolates present in agricultural ecosystems in Switzerland. The data obtained were combined with the results of earlier studies from 1992 to 1998. In total, data from more than 710 powdery mildew isolates tested on differential lines for the *Pm3a–Pm3d* alleles, from 494 isolates tested on *Pm3f* lines, and from 102 isolates for the *Pm3e* and *Pm3g* lines were available. We analysed if one of the *Pm3* alleles represents a natural example with an extended resistance spectrum compared with another *Pm3* gene. In this case, there would be no isolate showing virulence on the broad-spectrum allele and avirulence on the corresponding narrow-spectrum allele, and so we specifically searched for missing combinations of virulence/avirulence on the different *Pm3* alleles. We found numerous isolates avirulent on *Pm3a*, and either avirulent on *Pm3f* (*AvrPm3a/AvrPm3f*) or virulent on *Pm3f* (*AvrPm3a/avrPm3f*; note that 'avr' with a small 'a' indicates absence of the corresponding avirulence [Avr] factor), as well as isolates virulent on both alleles (Figure 1a; Table S1a). However, no isolates were found with the combination of avirulence on *Pm3f* and virulence on *Pm3a.* A similar pattern was observed for virulence/avirulence on the allelic pair *Pm3b* and *Pm3c,* where all powdery mildew isolates avirulent on *Pm3c* were also avirulent on *Pm3b*, and none were avirulent on *Pm3c* and virulent on *Pm3b* (*AvrPm3c/avrPm3b*; Figure 1b; Table S1b). These observations were confirmed by a re-examination of earlier publications describing powdery mildew infection tests on *Pm3* alleles. In these publications, isolates scored as avirulent on *Pm3f* or *Pm3c* were never virulent on *Pm3a* or *Pm3b* wheat lines, respectively (Briggle 1969, Huang *et al.* 2004, Huang and Röder 2004). Based on the frequencies of virulence on *Pm3a* (8.1%) and *Pm3b* (7.9%), and of avirulence on *Pm3f*

(35.0%) and *Pm3c* (47.5%) in our studied mildew population, we expected to observe 14 isolates virulent on *Pm3a* and avirulent on *Pm3f* (out of 494 isolates), and 27 isolates virulent on *Pm3b* and avirulent on *Pm3c* (out of 710 isolates). The absence of these (a)virulence combinations is very unlikely the result of statistical fluctuation (Chi-square test, $P < 0.001$ for both pairs), but can be explained by an extended resistance spectrum of *Pm3a* and *Pm3b* compared with *Pm3f* and *Pm3c*.

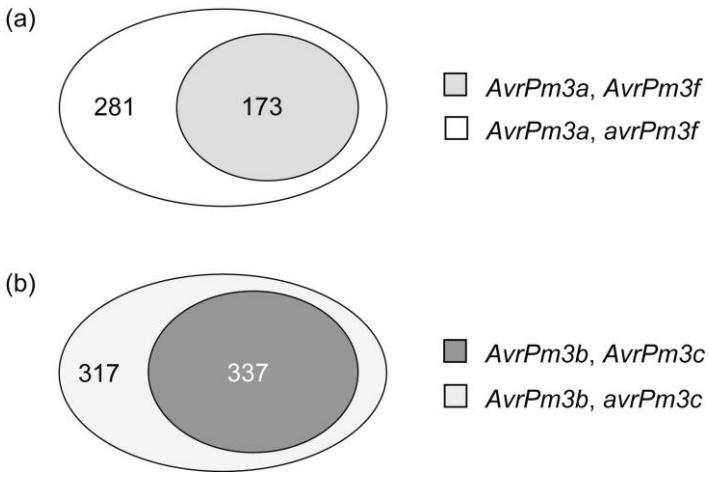

Figure 1. *Pm3a/Pm3f* and *Pm3b/Pm3c* avirulence gene combinations found in powdery mildew isolates.

(a) All 173 isolates avirulent on *Pm3f* were also avirulent on *Pm3a*, and thus are included in the total of 454 isolates avirulent on *Pm3a*.

(b) Similarly, all 337 isolates avirulent on *Pm3c* were also avirulent on *Pm3b*. No isolates were found that were virulent on *Pm3a* and avirulent on *Pm3f* (*avrPm3a/AvrPm3f*) or virulent on *Pm3b* and avirulent on *Pm3c* (*avrPm3b/AvrPm3c*).

2.3.2 Polymorphisms both in the ARC2 and LRR domains of PM3A are required for full PM3A-mediated resistance

We hypothesized that particular sequence segments in the allelic pairs *Pm3a*/*Pm3f* and *Pm3b*/*Pm3c* could be responsible for the shared resistance spectrum. We first analysed the sequence differences between the *Pm3a* and *Pm3f* alleles, which are more similar to each other than to any other *Pm3* allele. The encoded proteins PM3A and PM3F exclusively share, among all PM3 allelic variants, two residues each in LRRs 2, 13 and 14, and another five residues in LRR 27 (Figure 2a); with PM3B, they have in common two polymorphic residues in LRR 1 (Figure 2a) and a sequence block in the spacer region, which connects the NB-ARC and the LRR domain (Figure 2b). PM3A and PM3F differ from each other by two clearly delimited, polymorphic sequence blocks: one in LRRs 19-22 (13 amino acid differences) and one in the ARC2 domain and spacer region (19 amino acid differences in the ARC2, and one in the spacer). Therefore, we addressed the question of how these two segments determine the observed differences in the resistance spectra of the two alleles.

We constructed three chimeras between *Pm3a* and *Pm3f* (Figure 3). The first two, *Pm3a-f*$_{ARC}$ and *Pm3f-a*$_{ARC}$, were produced *in vitro* by exchanging reciprocally the ARC-encoding sequences between *Pm3a* and *Pm3f*. Similarly, the replacement of the LRR-encoding sequence of *Pm3a* with the corresponding sequence of *Pm3f* yielded *Pm3a-f*$_{LRR19-22}$. This third construct is identical to *Pm3f-a*$_{ARC}$, except for a threonine at position 543 (T_{543}) in the spacer region, which is shared with PM3A, but not with PM3F (Figures 2b and 3). These and all further constructs used in this study were functionally tested in a transient expression system (Schweizer *et al.* 1999), where they were biolistically delivered into leaf epidermal cells of the wheat line Chancellor that does not carry an endogenous copy of *Pm3* (Yahiaoui *et al.* 2004). The leaves were subsequently infected with a specific powdery mildew isolate and the infection level was quantified as

a haustorium index (HI) that gives the percentage of susceptible interactions in transformed cells. The *Pm3a*/*Pm3f* chimeras were tested with a powdery mildew isolate that distinguishes the reaction of *Pm3a* from *Pm3f.* Based on wheat seedling infection results, we chose isolate 97028, which is avirulent on *Pm3a* (HI of 17%) and significantly more virulent on *Pm3f* (49% HI; Student's *t*-test: $P < 0.001$; Figure 3). As a susceptible control, we used *Pm3CS* (76% HI), the naturally occurring susceptible *Pm3* allele that has the consensus sequence of the resistance alleles *Pm3a–Pm3g* and is equal to an empty vector control (Yahiaoui *et al.* 2006). All three chimeric constructs showed a resistance level intermediate between *Pm3a* and *Pm3f* (32, 24 and 25% HI; Student's *t*-test, $P < 0.01$; Figure 3). This indicates that: (i) the PM3A-specific sequence in both the ARC2 domain and the LRRs 19-22 contribute independently to the increased resistance of PM3A compared with PM3F, (ii) only their combination leads to the resistance level of PM3A, and (iii) that the single amino acid difference between PM3F-A_{ARC} and PM3A-$F_{LRR19\text{-}22}$ in the spacer region has no significant effect on the resistance level (Student's *t*-test, $P = 0.116$). As controls, we tested the chimeric constructs with powdery mildew isolates 96224 and 07201, which are avirulent and virulent, respectively, on both *Pm3a* and *Pm3f* (Figure 3). They were not significantly different from *Pm3a* and *Pm3f* when tested with isolate 96224 (Student's *t*-test, $P > 0.4$), nor from *Pm3CS* when tested with isolate 07201 (Student's *t*-test, $P > 0.06$). Thus, the conclusions above are not the result of artefacts like instability or autoactivity of the chimeric proteins.

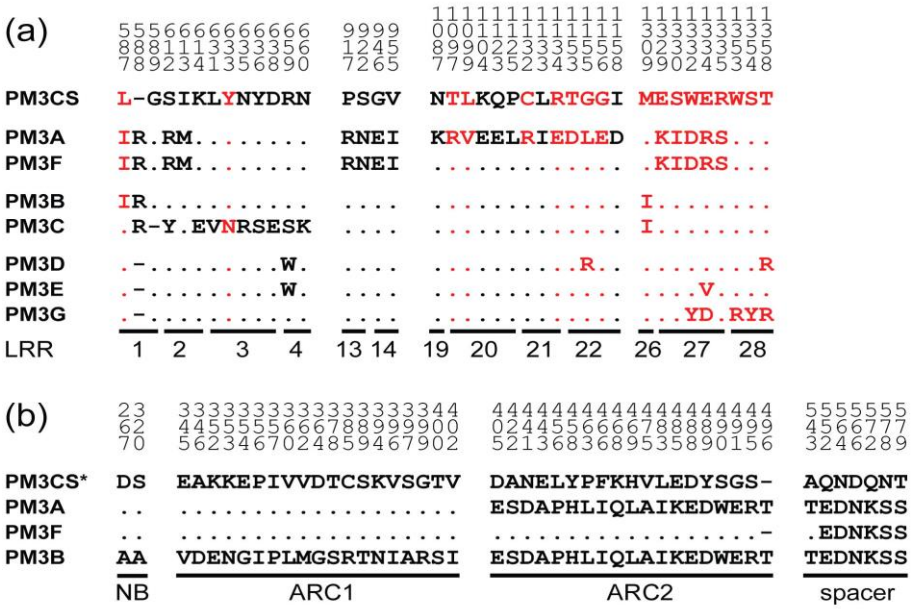

Figure 2. Amino acid polymorphisms in the PM3A–PM3G proteins.

(a) The first sequence (PM3CS) lists the consensus residues at the polymorphic sites of the leucine-rich-repeat (LRR) domain (vertical numbers give the amino acid positions in the PM3 protein). Below the PM3CS sequence, the polymorphic residues and the corresponding PM3 proteins are indicated. Dots represent residues identical to those in PM3CS and deletions are shown as dashes. Amino acids at the x-positions of the LxxLxLxx motif are highlighted in red. The LRR numbers are given underneath.

(b) Polymorphic sites in the NB-ARC domains of PM3 proteins are listed below the corresponding PM3CS residues. Spacer is the region connecting the NB-ARC domain with the LRR domain. *The NB-ARC and spacer sequences of PM3C–PM3E and PM3G are identical to PM3CS.

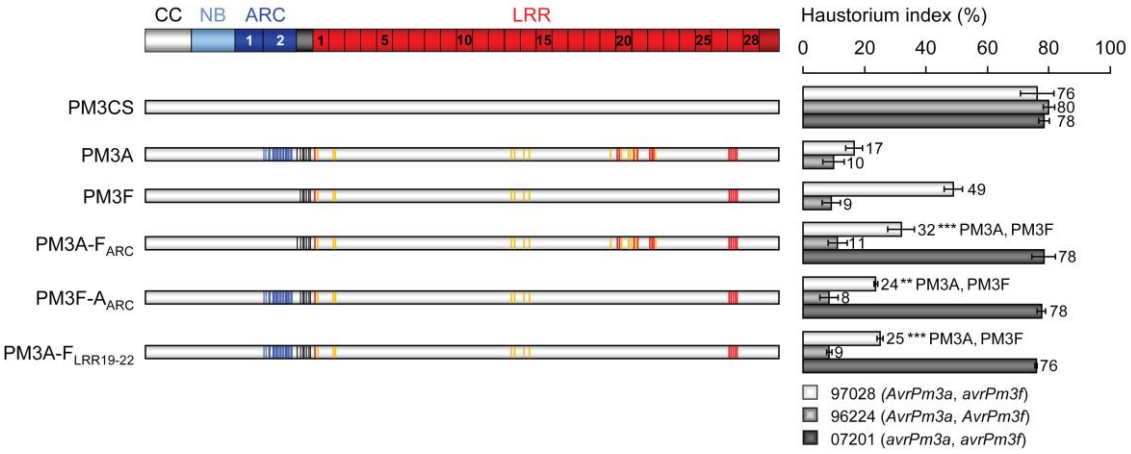

Figure 3. Polymorphisms both in the ARC2 domain and in leucine-rich-repeats (LRRs) 19-22 of PM3A are required for full PM3A-mediated resistance.

Left panel: Schematic diagram of wild-type and chimeric PM3 proteins. On top, the domain structure is indicated (not drawn to scale). Bars represent polymorphic amino acids compared with the consensus sequence PM3CS, which was used as susceptible control (blue bars, polymorphisms in the NB-ARC domain; black bars, polymorphisms in the spacer region; red bars, polymorphisms in the x-residues of the LxxLxLxx motif of the LRRs; yellow bars, all other polymorphisms in LRRs).

Right panel: Constructs encoding the proteins shown in the left panel were driven by the CaMV 35S promoter and tested by transient expression in susceptible wheat leaves. Leaves were biolistically transformed, challenged with a single powdery mildew isolate, and resistance responses were evaluated by microscopic analyses. The powdery mildew isolate used is indicated below the graph, and presence (*Avr*) or absence (*avr*) of the relevant avirulence genes, as deduced from leaf segment infection tests, is stated. The result is indicated as haustorium index, which gives the percentage of compatible interactions (values report the mean of three independent experiments and error bars give the standard deviation; for comprehensive statistical analysis see Table S2). The relevant significant differences at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ are indicated.

2.3.3 The NB-ARC domain of PM3 proteins controls the efficiency of effector-dependent resistance

Interestingly, the two broad-spectrum proteins PM3A and PM3B both have a distinct NB-ARC domain sharing an identical ARC2 sequence, while the NB-ARC domains encoded by *Pm3c–Pm3g* and *Pm3CS* are identical (Figure 2b). In particular, PM3A and PM3B share all polymorphic residues in the ARC2 domain. The latter protein has two additional amino acid differences in the NB, and 19 differences in the ARC1 domain compared with all other PM3 proteins. We wanted to investigate whether the characteristic NB-ARC domain of PM3B also contributes to the difference in recognition between PM3B and PM3C. Several powdery mildew isolates avirulent on *Pm3b* and virulent on *Pm3c* in seedling infection tests were avirulent on transiently transformed cells expressing *Pm3c*. We made a similar observation for *Pm3a* and *Pm3f*, where there was no isolate showing full virulence on *Pm3f* and avirulence on *Pm3a* in the transient assay (Figure 3). This might be because of the nature of the transient assay in which the genes are overexpressed. In fact, this result indicates that PM3C confers similar resistance specificity as PM3B when it is present at higher levels. Thus, it was not possible to directly relate the presence of the PM3B-specific NB-ARC to the functional difference of PM3B compared with PM3C. Instead, we performed domain-swap experiments using the PM3B NB-ARC domain to test its contribution to the resistance function of other *Pm3* genes and to the *Pm3b*-specific resistance.

We reciprocally exchanged the NB-ARC encoding region of *Pm3b* and *Pm3d* to construct the recombinant genes *Pm3b-d*$_{NB\text{-}ARC}$ and *Pm3d-b*$_{NB\text{-}ARC}$ (Figure 4a). These chimeras were functionally tested with the isolates 97011 (avirulent on *Pm3d*; virulent on *Pm3b*) and 96229 (avirulent on *Pm3b*; virulent on *Pm3d*), which discriminate *Pm3b*- from *Pm3d*-dependent resistance. Like *Pm3b*, *Pm3b-d*$_{NB\text{-}ARC}$ was not functional against isolate 97011. However, it was significantly less effective than *Pm3b* to isolate 96229

(40% HI compared with 11% HI; Student's *t*-test, $P < 0.01$). This indicates that polymorphisms in the NB-ARC of PM3B enhance the PM3B-dependent resistance response. The second construct, *Pm3d-b*$_{NB\text{-}ARC}$, showed significant quantitative differences compared with *Pm3d* (Student's *t*-test, $P < 0.05$): the resistance to 97011 was stronger (a HI reduction from 31 to 16%), and the susceptibility to 96229 was decreased from 82 to 53% HI. This result raises the question whether the PM3B-specific NB-ARC domain causes a weak autoactivation of PM3D. Therefore, we challenged leaves transiently expressing *Pm3d-b*$_{NB\text{-}ARC}$ with the isolate 07016, which is virulent on both *Pm3b* and *Pm3d*. This resulted in a HI of 77% (Figure 4a), demonstrating that *Pm3d-b*$_{NB\text{-}ARC}$ is not autoactive.

To further test the hypothesis of whether the PM3B NB-ARC domain enhances the resistance response, we made the construct *Pm3CS-b*$_{NB\text{-}ARC}$ by replacing the consensus NB-ARC domain of the susceptible protein PM3CS with the NB-ARC of PM3B. We tested it functionally by using isolate 96229 and an isolate avirulent on all seven alleles *Pm3a–Pm3g* (96224; Figure 4b). *Pm3CS-b*$_{NB\text{-}ARC}$ did not confer resistance to either of the isolates. This indicates that the NB-ARC domain of PM3B does not contribute to the recognition of isolate 96229 (and 96224). The lower HI of PM3B compared with PM3B-D$_{NB\text{-}ARC}$, and of PM3D-B$_{NB\text{-}ARC}$ compared with PM3D, upon challenge with isolate 96229 (Figure 4a) can be explained by a higher resistance protein activity caused by the PM3B NB-ARC domain. It is likely that the LRR domain of PM3D (but not of PM3CS; Figure 4b) weakly senses the presence of isolate 96229, but that the presence of the PM3B NB-ARC in PM3D-B$_{NB\text{-}ARC}$ activates the protein sufficiently to allow the triggering of a (still weak) resistance response. A role of the PM3B NB-ARC in the activity, but not the resistance specificity, of PM3 proteins is also consistent with the increased resistance of PM3D-B$_{NB\text{-}ARC}$ compared with PM3D to isolate 97011 (Figure 4a). Considering the sequence similarity of the NB-ARC in PM3B

and PM3A (Figure 2b), it is tempting to speculate that PM3A NB-ARC also increases the resistance activity, causing the reduced HI of PM3A compared with PM3A-F_{ARC}, and of PM3F-A_{ARC} and PM3A-$F_{LRR19-22}$ compared with PM3F, when challenged with isolate 97028 (Figure 3).

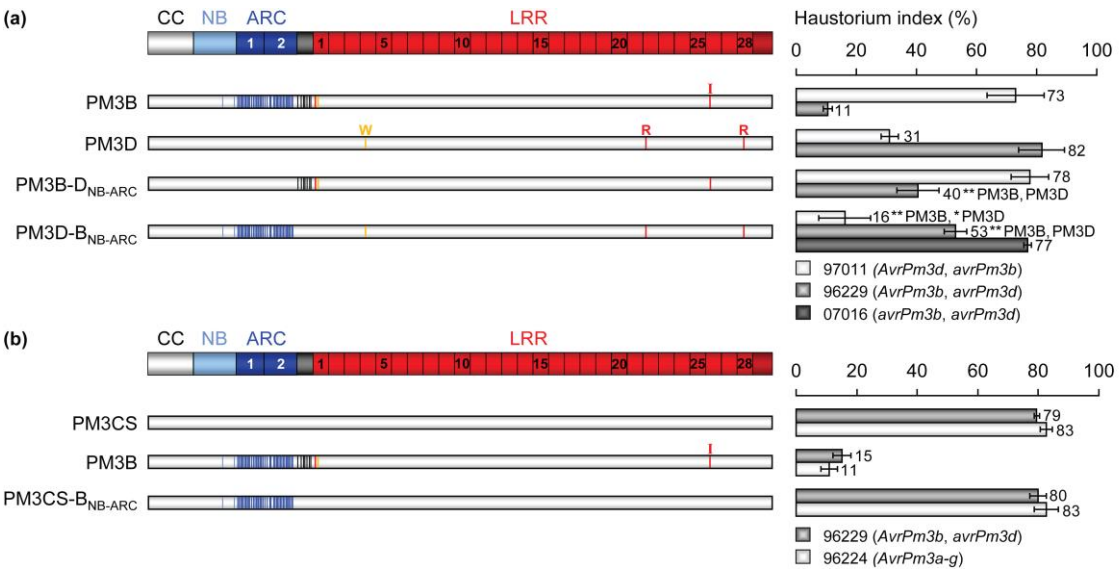

Figure 4. Polymorphisms in the NB-ARC domain of PM3B enhance resistance activity.
(a) Chimeric constructs of *Pm3b* and *Pm3d* were transiently expressed in wheat and tested for resistance against powdery mildew isolates 97011 and 96229, which discriminate *Pm3b*- from *Pm3d*-dependent resistance. The construct *Pm3d-b*$_{NB-ARC}$ was also challenged with a virulent control isolate (07016).
(b) A domain swap between *Pm3b* and *Pm3CS* was functionally analysed using isolates 96229 and 96224.
The most relevant polymorphic residues are specified by the single letter code above the drawings. The designations and experimental procedures are the same as described in Figure 3. Values report the mean of three (isolates 97011 and 96229) or two (isolate 07016) independent experiments, and error bars give the standard deviation. For comprehensive statistical analysis see Tables S3, S4, and S5.

2.3.4 Modelling polymorphic residues of PM3A and PM3B in the NB-ARC domain structure

To better understand the impact of the amino acid polymorphisms between the PM3A/B- and the PM3CS-type of NB-ARC domains, we determined their position relative to conserved sequences in other R proteins. Therefore, the consensus sequence PM3CS was added to the structure-based multiple sequence alignment of the NB-ARC domains of different R proteins published in van Ooijen *et al*. (2008b). On PM3CS, the positions of the polymorphic amino acids present in PM3A and/or PM3B were marked (Figure S1). Only four of them aligned to residues conserved in the majority of the R proteins, and the underlying substitutions in PM3A/PM3B were conservative ($V_{362}M$, $V_{396}I$, $T_{400}S$, $F_{466}I$). Furthermore, the polymorphic positions do not map to gain- and loss-of-function positions described in the other R proteins, nor corresponded to positions predicted to be involved in ADP binding. Based on the alignment, we constructed a protein structure model of PM3CS using the crystal structure of human APAF-1 (Riedl *et al.* 2005) as a template in order to find the 3D position of polymorphic sites in the protein. Alignment gaps were substituted by loop modelling of PM3CS. Remarkably, polymorphic sites are evenly distributed in the ARC1 (Figure 5a), whereas 16 of the 18 variant amino acids located in the ARC2 cluster on one side of the subdomain (Figure 5b). Their side chains point to the outside of the subdomain, with only three exceptions (P463, F466, S495), and two side chains have interdomain contacts with the NB domain (L483, E485). Furthermore, there are two loops in the PM3CS sequence that are considerably longer compared to APAF-1, and six polymorphic sites locate there. These observations derived from the protein structure model of PM3CS indicate an important role of one side of the ARC2 subdomain for molecular interaction and signalling.

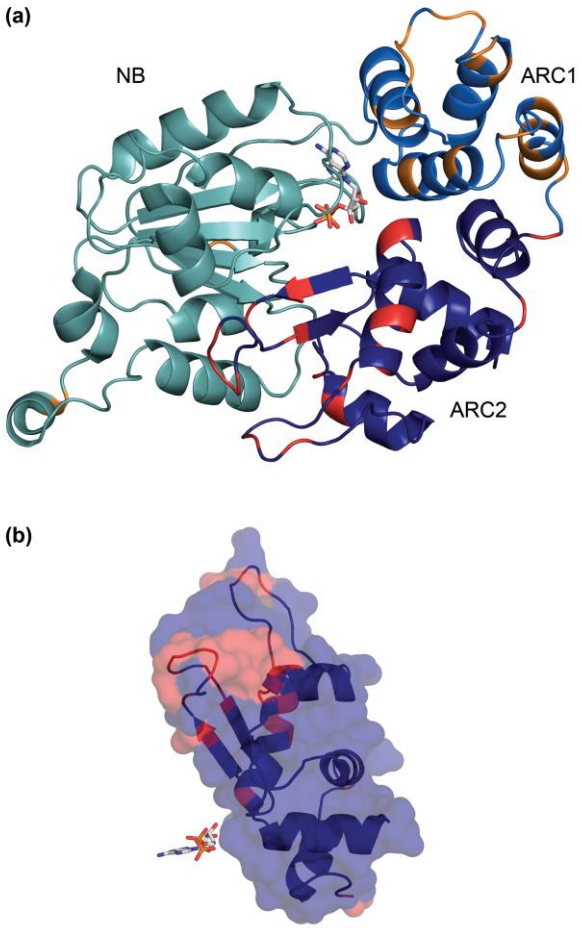

Figure 5. Polymorphic amino acids of PM3A and PM3B locate mostly on one side of the ARC2 subdomain.

A protein structure model of the PM3CS sequence was constructed to localize variant amino acids in 3D. Residues that correspond to polymorphic amino acids in PM3B are indicated in orange; those present in both PM3A and PM3B are highlighted in red. The NB domain is coloured in cyan, the ARC1 subdomain is coloured in marine and the ARC2 subdomain is coloured in dark blue. Bound ADP is represented as sticks in CPK atom colours.

(a) View of the complete NB-ARC domain. Variant amino acids are evenly spread in the ARC1 subdomain.

(b) Alternative view of the ARC2 subdomain with transparent surface visualisation to show the 3D distribution of polymorphic sites. Of 18 polymorphic sites, 16 locate to one side of the subdomain, and only three of those are completely buried.

2.3.5 Polymorphic residues in both the N- and C-terminal LRRs of PM3B, PM3C and PM3D are necessary for resistance specificity

PM3A and PM3F have identical sequences both in the N-terminal and the C-terminal part of the LRR domain (Figure 2a). PM3B and PM3C differ in LRRs 1-4, but share the polymorphic residue I_{1309} in LRR 26 compared with PM3CS. In contrast, the remaining PM3 proteins show unique polymorphic residues in the C-terminal part of the LRR (LRRs 26–28). Thus, only the allelic pairs *Pm3a/Pm3f* and *Pm3b/Pm3c* encode identical C-terminal LRRs. We hypothesised that this sequence identity is the basis of their overlap in resistance spectrum. Therefore, we studied the dependence of PM3B and PM3C function on the shared polymorphic residue I_{1309}. *Pm3b* and *Pm3c* were recombined with *Pm3CS*, resulting in the constructs *Pm3b-CS*$_{LRR26}$, *Pm3c-CS*$_{LRR26}$ and *Pm3CS-b/c*$_{LRR26}$ (Figure 6a). These constructs were transiently expressed and the transformed cells were challenged with the powdery mildew isolates 96224 and, partly, 97019, which are avirulent on *Pm3b* and *Pm3c* (Figure 6a). *Pm3b-CS*$_{LRR26}$ and *Pm3c-CS*$_{LRR26}$ were both partially compromised in resistance function. The residual resistance activity was effector-dependent, as they were not significantly different from *Pm3CS* (Student's *t*-test, $P > 0.2$) when challenged with the virulent isolates 07016 and 07296, respectively. *Pm3CS-b/c*$_{LRR26}$ also showed partial resistance to 96224 (49% HI), but full susceptibility to 97019 (85% HI; Figure 6a). These results demonstrate that I_{1309} is functionally important in both PM3B and PM3C. Its replacement in PM3C and PM3B by the conserved methionine of PM3CS leads to a decreased resistance level, but I_{1309} alone (construct *Pm3CS-b/c*$_{LRR26}$) is not sufficient to confer complete *Pm3b*- or *Pm3c*-dependent resistance. In PM3C-CS$_{LRR26}$, the residual recognition capacities are the result of sequence polymorphisms in the N-terminal LRRs. One of these residues, R_{588}, is also shared by PM3B, as well as by PM3A and PM3F (Figure 2a). As we have previously shown that the NB-ARC domain does not contribute to the recognition of isolate 96224 (Figure 4b), the residual recognition capacities of PM3B-CS$_{LRR26}$ (Figure

6a) must result from the two polymorphisms in LRR 1 and/or polymorphic residues in the spacer region.

We conclude that polymorphic amino acids at both N- and C-terminal ends of the LRR are necessary to confer full *Pm3c*- and possibly also *Pm3b*-specific resistance. This is reminiscent of our previous studies on PM3D, which was also functionally dependent on polymorphisms in both C- and N-terminal LRRs (Yahiaoui *et al.* 2006). In these studies, both the replacement of the only polymorphic residue in the N-terminal LRRs (W_{659} in LRR 4), or the replacement of C-terminal polymorphisms (R_{1155} in LRR 22 and R_{1358} in LRR 28) by conserved residues of PM3CS led to a complete loss of resistance function. To further analyse the role of amino acid polymorphisms in the N- and C-terminal LRRs of PM3 proteins, we reciprocally exchanged the last eight LRRs of PM3B and PM3D in which they differ by three amino acids in the LRRs 22, 26 and 28 (Figure 6b). The resulting constructs, *Pm3b-d*$_{LRR22,28}$ and *Pm3d-b/c*$_{LRR26}$, failed to confer resistance to two tested isolates, 97011 and 96229. The chimera *Pm3b-d*$_{LRR22,28}$ showed residual resistance activity (40% HI) to isolate 96229, similar to *Pm3b-CS*$_{LRR26}$, which also caused a weak resistance response to isolate 96224 (Figure 6a). Also in this case, the residual resistance was effector-dependent, as *Pm3b-d*$_{LRR22,28}$ showed full susceptibility to isolate 97011, and is most probably mediated by polymorphisms in the spacer region and/or the N-terminal LRRs of the PM3B-protein. The failure of *Pm3b-d*$_{LRR22,28}$ to mediate resistance to isolate 97011 confirms that *Pm3d*-dependent resistance depends on both N-terminal and C-terminal residues of the LRR domain.

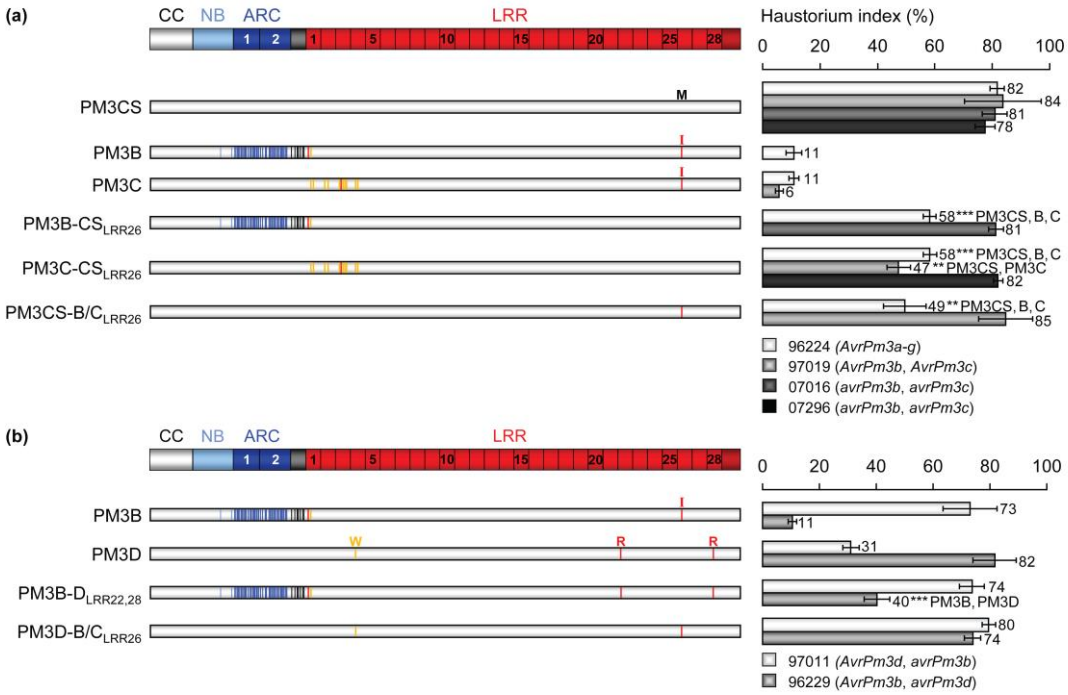

Figure 6. Polymorphic residues in both C- and N-terminal LRRs contribute to *Pm3c*- and *Pm3d*-specific resistance.

(a) The residue I_{1309} plays a similar role in PM3B- and PM3C-mediated resistance.

(b) The swapping of the C-terminal LRRs of PM3B and PM3D disrupts their resistance specificity.

Note that the wild-type controls *Pm3b* and *Pm3d* in (b) are identical to those in Figure 4(a), as *Pm3b*, *Pm3d*, *Pm3b-d*$_{NB\text{-}ARC}$, *Pm3d-b*$_{NB\text{-}ARC}$, *Pm3b-d*$_{LRR22,28}$ and *Pm3d-b/c*$_{LRR26}$ were tested in parallel. The designations and experimental procedures are the same as described in Figure 3. Values report the mean of three (isolates 96224, 97019, 97011 and 96229) or two (isolates 07016 and 07296) independent experiments, and error bars give the standard deviation. For comprehensive statistical analysis see Tables S4, S6 and S7.

2.3.6 Intragenic allele pyramiding of *Pm3d* and *Pm3e* leads to a functional gene with dual resistance specificities

We hypothesized that it should be possible to generate a gene with multiple recognition specificities by combining polymorphic residues of different functional alleles residing in the C-terminal LRR domain. To test if such pyramiding is possible in principle, we considered only combinations of *Pm3* alleles that: (i) have identical NB-ARC sequences to avoid possible interfering effects of this domain; (ii) functionally identical N-terminal LRRs (ideally identical in sequence); and (iii) have the polymorphic amino acids in different LRRs to circumvent sterical changes that might inhibit proper folding or alter a putative interaction surface. The alleles *Pm3d* and *Pm3e* fulfilled these conditions: PM3D and PM3E differ from each other by only three amino acids in the C-terminal LRRs 22, 27 and 28 (Figure 2). We made the construct *Pm3d+e* that combines all polymorphic sites of PM3D and PM3E compared with PM3CS (Figure 7a). Its function was tested by transient transformation and challenged with the differential isolates 97019 (avirulent on *Pm3e*: virulent on *Pm3d*) and DB Asosan (avirulent on *Pm3d*; virulent on *Pm3e*). Results of this assay showed that the chimera PM3D+E conferred resistance to both isolates at the same level as the original PM3D and PM3E (Figure 7a). The control experiment with the virulent isolate 94202 confirmed that the resistance mediated by *Pm3d+e* was effector dependent.

To confirm the dual function of the Pm3d/Pm3e-pyramid, we stably transformed wheat line Bobwhite SH 98 26, which does not carry an endogenous *Pm3* copy, with *Pm3d+e* under the control of the maize ubiquitin promoter. In two independent, segregating T_1 and T_2 families, presence of the transgene co-segregated with the resistance to the previously used isolates 97019 (virulent on line Kolibri carrying *Pm3d*; avirulent on line W150 carrying *Pm3e*), and to DB Asosan (avirulent on Kolibri; virulent on W150), as inferred from Southern blot analysis and leaf segment infection tests (Figure 7b). All

tested plants showed susceptibility to isolate 94202, demonstrating that PM3D+E is not autoactive, but confers race-specific resistance (Figure 7b). The T_2 generation was resistant when challenged with the isolates 09003 (virulent on Kolibri; avirulent on W150) and Ken 2-5 (avirulent on Kolibri; virulent on W150), thus confirming the results with the isolates 97019 and DB Asosan. The reproducibility of the transient expression assay results (Figure 7a) in stable transgenic plants demonstrates that *Pm3d+e* indeed represents a functional allele pyramid. Thus, based on the detailed analysis of the functional role of individual subdomains of the PM3 protein by a series of domain-swap experiments, it was possible to successfully predict a chimeric allele with pyramided resistance specificities.

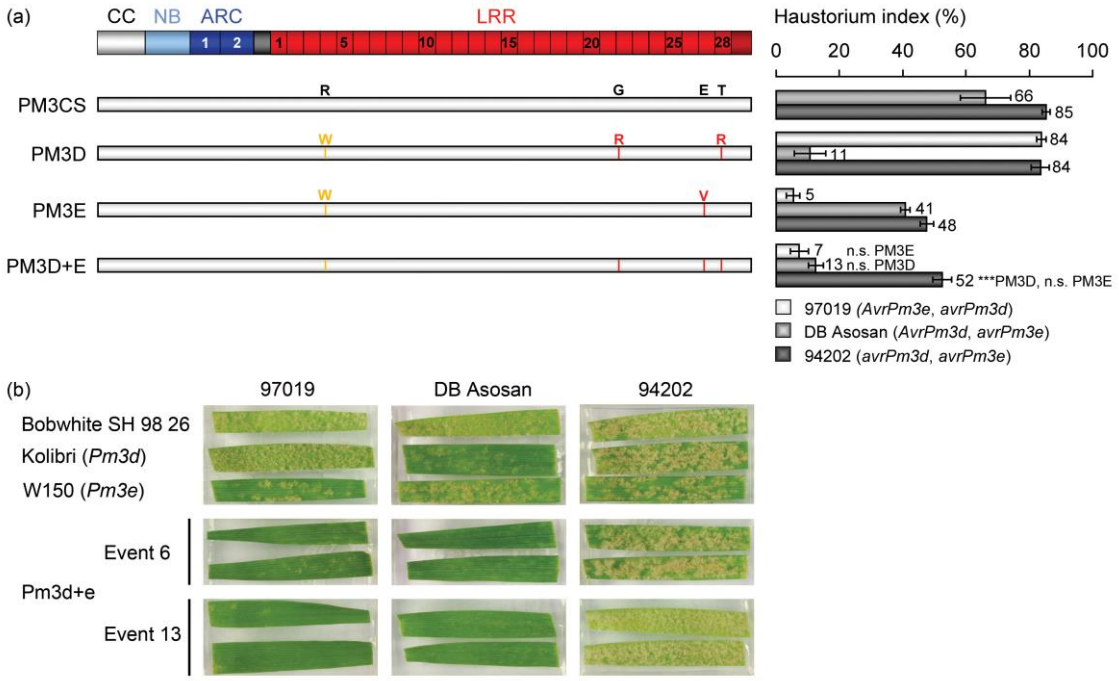

Figure 7. Intragenic allele pyramiding resulted in construct *Pm3d+e*, which conferred both *Pm3d*- and *Pm3e*-dependent resistance.

(a) In the transient expression assays, the haustorium index of hybrid construct *Pm3d+e* was not significantly different (n.s.; Student's *t*-test, $P > 0.4$) from the one of *Pm3e* after infection with isolates 97019 and 94202, and from the one of *Pm3d* after infection with isolate DB Asosan. The designations and experimental procedures are the same as described in Figure 3. Values report the mean of three independent experiments and error bars give the standard deviation. For comprehensive statistical analyses see Table S8.

(b) Representative pictures of infection phenotypes of leaf segments from control lines (Bobwhite SH 98 26, Kolibri, W150) and transgenic T_2 *Pm3d+e* plants infected with powdery mildew isolates 97019, DB Asosan and 94202. For Pm3d+e, two leaf segments of each of the two independent transformation events 6 and 13 are shown.

2.4 Discussion

2.4.1 Virulence analyses in pathogen populations detect natural examples of *Pm3* alleles with enlarged recognition spectra

We used a large set of powdery mildew isolates to characterize the resistance spectrum of *Pm3* alleles. These studies indicated that *Pm3a* and *Pm3b* represent alleles with an extended resistance spectrum compared with *Pm3f* and *Pm3c*, respectively. Studies in flax showed that the flax rust resistance genes *L5*, *L6* and *L7* have overlapping resistance specificities, which are based on the recognition of the same *Avr* gene, *AvrL567* (Dodds *et al.* 2004, Dodds *et al.* 2006). L6-mediated resistance is more effective (no fungal growth) than L7-dependent resistance (low level of rust sporulation), and in contrast to L7, L6 is not suppressed by a fungal inhibitor, because of the L6-specific polymorphisms in the TIR domain (Dodds *et al.* 2006, Luck *et al.* 2000). *L5* shows a reduced recognition repertoire compared with *L6* and *L7*, but the underlying molecular basis is not yet described. However, recombinant *L* alleles showed novel resistance specificities by the loss of recognized rust strains, compared with the parental alleles. These specificities are determined by one or two amino acids in a C-terminal LRR (L6L11RV; Dodds *et al.* 2006), or by five or fewer amino acids in the TIR-NB domains (RL10-1, RL10-2/3, L2-L10Sph; Ellis *et al.* 1999, Luck *et al.* 2000).

The study of the *Pm3* alleles with enlarged resistance spectra allowed us to identify determinants of different powdery mildew isolate recognition spectra, and to propose hypotheses on the underlying molecular mechanism. We found that the NB-ARC domains in PM3B and PM3A increase resistance activity. The PM3A-specific sequence in C-terminal LRRs was shown to contribute to the higher resistance of PM3A compared with PM3F, possibly by contributing to a higher binding affinity to AvrPM3A. On the pathogen side, the quantitative difference in resistance intensity within the *Pm3a*/*Pm3f* and *Pm3b*/*Pm3c* pairs might be caused by slightly different biochemical

properties of the different AVR proteins, resulting in differential binding affinities. The cloning of the *AvrPm3* genes will offer the prospects of testing these hypotheses experimentally.

2.4.2 The ARC2 domain modulates PM3 activity

The results of constructs with reciprocally swapped NB-ARC domains of PM3A and PM3B point to a role of the NB-ARC domain in controlling the efficiency of effector-dependent resistance. PM3A and PM3B share all polymorphic residues in the ARC2 domain. As both NB-ARC domains cause a comparable HI reduction (*Pm3a* compared with *Pm3a-f*$_{ARC}$, and *Pm3f-a*$_{ARC}$ and *Pm3a-f*$_{LRR19-22}$ compared with *Pm3f*, Figure 3; *Pm3b* compared with *Pm3b-d*$_{NB-ARC}$, and *Pm3d-b*$_{NB-ARC}$ compared with *Pm3d*, Figure 4a), it is likely that the polymorphisms in the ARC2 domain specifically cause this alteration. The ARC2 domain of R proteins was described as the regulatory element that transduces pathogen perception by the LRR domain into R-protein activation (Tameling *et al.* 2006). It was suggested that the LRR domain binds directly to the ARC1 domain, with an alteration of this interaction upon effector binding that would be transmitted by the ARC2 domain (Rairdan and Moffett 2006, van Ooijen *et al.* 2008a). A 3D structural model shows that the PM3A- and PM3B-specific amino acids locate mainly on one side of the ARC2 subdomain (Figure 5b). Together with the predominant position of the variant residues within long loops and on the surface, our results suggest an important role of that region for interdomain interactions, facilitating changes in the LRR-ARC1 interaction. Alternatively, these polymorphic residues could also increase the affinity with other factors required for downstream signalling or stabilize a certain protein conformation required for signalling activity or for pathogen perception, leading to a signal more intense in time and/or amplitude.

2.4.3 The role of the N- and C-terminal LRRs in PM3 race specificity

Pathogen recognition specificity of PM3 proteins is determined by their LRR domain. This was also found in other LRR-containing plant R proteins (e.g. Dodds *et al.* 2001, Ellis *et al.* 2007, Ellis *et al.* 1999, Rairdan and Moffett 2006, Shen *et al.* 2003, Wulff *et al.* 2009, Wulff *et al.* 2001, Zhou *et al.* 2006). Additional sequences outside the LRR domain were also reported to be involved in recognition specificity (Luck *et al.* 2000). Studies on some mammalian NACHT-LRR proteins (NLPs) have revealed two different roles of the LRR domain: the N-terminal LRRs modulate activation, whereas C-terminal LRRs are responsible for bacterial recognition (Inohara and Nunez 2003, Tanabe *et al.* 2004). Possibly, these different subdomain functions are conserved in plant NB-ARC-LRR proteins (Belkhadir *et al.* 2004, Lukasik and Takken 2009). Domain swaps of flax *L6*/*L11*, barley (*Hordeum vulgare*) *Mla1*/*Mla6* and potato *Rx*/*Gpa2*, as well as mutational analysis of *Rx*, have shown that recognition specificity is determined by C-terminal LRRs alone (*L6*, *Mla6*), or may involve also N-terminal LRRs (Ellis *et al.* 2007, Farnham and Baulcombe 2006, Rairdan and Moffett 2006, Shen 2003). In PM3, there is evidence for a major role of the C-terminal LRRs in recognition specificity. Sequence analysis and our previous experiments (Yahiaoui *et al.* 2006) revealed that *Pm3e* and *Pm3g* specificity is determined exclusively by putative solvent-exposed residues of the LxxLxLxx motif in the C-terminal LRRs. Our domain-swap experiments showed that polymorphic residues in the LRRs 19-22 are required for *Pm3a*-specific resistance (Figure 3). However, PM3B, PM3C (this study) and PM3D (Yahiaoui *et al.* 2006) also depend functionally on the N-terminal LRR polymorphisms. In addition, polymorphic residues in the N-terminal LRRs of PM3B and PM3C confer residual, race-specific, resistance responses. In these two proteins, both N- and C-terminal LRRs might contribute to the recognition-mediating molecular interactions. Finally, it should be noted that a functional combination of N- and C-terminal LRRs also needs a fitting

ARC2 domain for optimal function: PM3B-D_{NBARC} and PM3D-B_{NBARC} are still functioning race-specifically, but they lose full resistance activity. This is reminiscent of findings from the tobacco N-like proteins (Gao *et al.* 2007).

The majority of the LRR domains (mainly from bacterial, animal or human proteins) that have been co-crystallized with their ligands show binding in the concave LRR face (Bella *et al.* 2008). In the PM3 proteins studied, polymorphic residues in the C-terminal LRRs occur exclusively in the non-conserved x-positions (marked in Figure 2a) of the putative LxxLxLxx motif on the predicted concave surface of the LRR domain. The only exception are polymorphic amino acids in the sequence block of LRRs 19–22 in PM3A, which is thought to be of ancient origin and derived from gene conversion (Yahiaoui *et al.* 2006). We consider it likely that these polymorphic x-positions in C-terminal LRRs interact directly with corresponding AVR proteins. This hypothesis is supported by the high level of diversifying selection in the PM3 proteins (Yahiaoui *et al.* 2006), which is assumed to be characteristic for direct protein-protein interactions (Wang *et al.* 2007). In the case of PM3B and PM3C, it is possible that polymorphic residues in the N-terminal LRRs are also involved in AVR binding, similar to the structural model for the flax AvrL567/L5 interaction, where the N- and C-terminal LRRs of L5 contribute to the AVR binding (Wang *et al.* 2007).

2.4.4 Intragenic pyramiding of *Pm3* allelic resistance specificities: towards artificial evolution of broad spectrum resistance

LRR-mediated ligand binding appears to involve single residues on the concave LRR surface that function in a cooperative manner (Bella *et al.* 2008, Herrin *et al.* 2008, Velikovsky *et al.* 2009). In flax L5 and L6, single amino acids were shown to have quantitative and qualitative effects on the R protein – Avr protein interaction (Wang *et al.* 2007). Based on these results, a model of stepwise evolution of *R* and *Avr* genes

was proposed. It states that a new *R* gene might evolve from a gene by an initial mutation that confers weak resistance. Subsequently, the accumulation of further mutations would lead to a strong resistance response. Our data suggest that such a stepwise evolution led to the extended, broad-spectrum resistance of *Pm3a* and *Pm3b* compared with *Pm3f* and *Pm3c*, respectively.

The flax *L5*/*L6*/*L7* alleles and the *Pm3a*/*Pm3f* and *Pm3b*/*Pm3c* alleles represent natural examples for broad- and narrow-spectrum *R* genes. In *Arabidopsis thaliana*, *RPP1-WsB* detects four alleles of the *Hyaloperonospora parasitica* avirulence gene *ATR1*, while its paralog *RPP1-Nd* recognizes only one of them (Rehmany *et al.* 2005). Recombinant *L* alleles of flax were reported to confer narrower recognition spectra compared with the parental alleles (Ellis *et al.* 2007, Ellis *et al.* 1999, Luck *et al.* 2000), and recombination between homologues of *Cf4/9* (tomato) and between paralogues of *Rp1* (maize) created novel resistance specificities (Parniske *et al.* 1997, Smith and Hulbert 2005). A first example for an artificial gene with broadened resistance spectrum comes from the mutational analysis of Rx, where in three cases a single amino acid change led to a broader resistance against potato virus X, and against a distantly related poplar mosaic virus (Farnham and Baulcombe 2006). The pyramiding of *Pm3d* and *Pm3e* now represents an example for a designed *R* gene with broader recognition spectrum. Thus, the molecular analysis of R proteins with overlapping spectra of specificity might lead to an improved understanding of the evolution of broad-spectrum resistance. The ultimate applied goal of such work would be the possibility to rationally design broad-spectrum *R* genes.

2.5 Experimental Procesures

2.5.1 Fungal strains

Our wheat powdery mildew (*B. graminis* f.sp. *tritici*) isolates originate from the former mildew collections of Agroscope Reckenholz-Tänikon ART (http://www.art.admin.ch) and INRA Rennes (http://www.rennes.inra.fr), from USDA-ARS, North Carolina State University, Raleigh (http://www.ars.usda.gov/saa/psru) and from our own isolate collection. The (a)virulences relevant for transient expression assays and seedling infection experiments are listed in Table 1.

Table 1 Powdery mildew isolates used for seedling infection experiments and transient expression assays

Isolate	Avirulence[a]	Virulence[b]
07016	-	*avrPm3b, avrPm3c, avrPm3d*
07201	-	*avrPm3a, avrPm3f*
07296	-	*avrPm3b*, *avrPm3c*
09003	*AvrPm3e*	*avrPm3d*
94202	-	*avrPm3d*, *avrPm3e*
96224	*AvrPm3a-Pm3g*	-
96229	*AvrPm3b*	*avrPm3d*
97011	*AvrPm3d*	*avrPm3b*
97019	*AvrPm3b, AvrPm3c, AvrPm3e*	*avrPm3d*
97028	*AvrPm3a*	*avrPm3f*
DB Asosan	*AvrPm3d*	*avrPm3e*
Ken 2-5	*AvrPm3d*	*avrPm3e*

[a] For each isolate, only the avirulence (*Avr*) genes relevant for this work are listed.

[b] For each isolate, only the absent avirulence genes (*avr*) relevant for our work are listed.

2.5.2 Wheat powdery mildew virulence profiling

Powdery mildew isolates were collected in the seasons 1992-1998 and in 2007 in the Swiss plateau with a spore trap, and single colony derived isolates were propagated and stored on leaf segments, as previously described (Winzeler *et al.* 1991). Virulence

for *Pm3a–Pm3g* was tested on a differential set of wheat lines and varieties using Asosan/8*Chancellor for *Pm3a*, Chul/8*Chancellor for *Pm3b*, Sonora/8*Chancellor for *Pm3c*, Kolibri for *Pm3d*, W150 for *Pm3e* (only tested in 2007), Michigan Amber/8*Chancellor for *Pm3f* (tested as of 1994) and Aristide for *Pm3g* (only in 2007). Wheat lines and varieties were grown and infected with the test isolates as described in Limpert *et al*. (1987). Three leaf segments on different plates were tested per line and isolate.

2.5.3 Construction of recombinant genes

The *Pm3a–Pm3g* and *Pm3CS* alleles have been cloned previously into the expression vector PGY1 (35S promoter and terminator; Srichumpa *et al.* 2005, Yahiaoui *et al.* 2006, Yahiaoui *et al.* 2004). All chimeric genes were constructed directly in PGY1 by using the unique restriction sites *Afl*II, *Bcl*I or *Nsi*I indicated in Figure S2, and the flanking sites *Bam*HI (5' end) or *Sal*I (3' end), or by site-directed mutagenesis (construct *Pm3d+e*), as described in Appendix S1. All constructs were checked by restriction enzyme digests and DNA sequencing of allele specific regions and junction sites.

2.5.4 Transient expression assay

Seven-day-old primary leaves of the susceptible wheat variety Chancellor were used for transient transformation. Particle bombardment was performed with the Biolistic PDS-1000/He System with the Hepta Adapter (Bio-Rad, http://www.bio-rad.com), following an adapted protocol of Duchkov *et al.* (2005). Per shot, 3 mg of gold particles (1-µm diameter; Bio-Rad) were coated with a mixture of 1.25 µg of pUbiGUS reporter plasmid (Schweizer *et al.* 1999) and 1.25 µg of the test plasmids (*Pm3* wild-type or recombinant genes in PGY1). Four hours after bombardment, leaves were infected with powdery mildew at high density and kept for 44 h on plates with slightly open lids

at 20°C, 16 h of light and 80% relative humidity. GUS staining (Schweizer *et al.* 1999), staining of the fungus with Coomassie blue (Schweizer *et al.* 1993) and microscopic evaluation (Yahiaoui *et al.* 2006) were performed as described previously.

2.5.5 In silico analysis

Sequence alignments were computed using MUSCLE (http://www.drive5.com/muscle; Edgar 2004). Shading of physicochemically conserved residues was produced by GeneDoc (http://www.psc.edu/biomed/genedoc). The secondary structure of PM3CS was predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred). The secondary structure assignment of the PDB structure of APAF-1 (PDB identifier 1z6t, chain A) was obtained from the DSSP database (http://www.cmbi.kun.nl/gv/dssp). Structure-based protein sequence alignment was constructed including the PM3CS sequence as described in van Ooijen *et al*. (2008b). A protein structure model of PM3CS was obtained by submitting the pairwise alignment of PM3CS and APAF-1 to the HOMER-M web server (http://protein.bio.unipd.it/homer). Alignment gaps were substituted by loop modelling of PM3CS sequence positions 453-462 (GFILEYKEDS) and 486-491 (SKDYSG), as well as including positions 357, 358, 389, 390 into the structure using the ModLoop web service (http://modbase.compbio.ucsf.edu/modloop; Fiser and Sali 2003). The protein structure image of the model including the positions of polymorphic sites was illustrated using PyMOL (http://www.pymol.org).

2.6 Acknowledgements

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2.7 Supporting Information

Appendix S1. Supplementary text for Experimental Procedures.

DNA manipulation for the construction of recombinant genes

Constructs *Pm3a-f*$_{ARC}$ and *Pm3f-a*$_{ARC}$ were made by reciprocally exchanging the LRR-encoding sequence between the restriction sites *Nsi*I and *Sal*I of *Pm3f* or *Pm3a*, respectively, with the corresponding fragment of *Pm3a* or *Pm3f*, respectively. The exchange of the LRR-encoding sequence between the restriction sites *Bcl*I and *Sal*I of *Pm3a* with the corresponding sequence of *Pm3f* yielded construct *Pm3a-f*$_{LRR19-22}$. Restriction double digests using *dam* methylation sensitive enzyme *Bcl*I combined with *Sal*I was performed on plasmid DNA isolated from *dam*$^{-}$/*dcm*$^{-}$ *E. coli* (New England Biolabs, http://www.neb.com). The chimeric genes *Pm3b-d*$_{NB-ARC}$ and *Pm3d-b*$_{NB-ARC}$ were generated by reciprocally exchanging *Nsi*I/*Sal*I fragments from *Pm3b* and *Pm3d*. This restriction site combination was also used to make *Pm3CS-b*$_{NB-ARC}$ with the templates *Pm3b* and *Pm3CS*. A reciprocal exchange of the *Bam*HI/*Afl*II fragments of *Pm3b* or *Pm3c* and *Pm3CS* yielded the constructs *Pm3b-CS*$_{LRR26}$, *Pm3c-CS*$_{LRR26}$ and *Pm3CS-b/c*$_{LRR26}$. Similarly, the *Bam*HI and *Afl*II sites of *Pm3b* and *Pm3d* were used for the generation of *Pm3b-d*$_{LRR22,28}$ and *Pm3d-b/c*$_{LRR26}$. The construct *Pm3d+e* was made using the *Pm3d* sequence as template into which the single amino acid change (from glutamate to valine at amino acid position 1332) was introduced with the primer pair Pm3_E1332V_F (5'-GGAATCCCTTTGGCTTG**T**AAGATGCAGTACCCTGG-3') and Pm3_E1332V_R (5'-CCAGGGTACTGCATCTT**A**CAAGCCAAAGGGATTCC-3') following the manual of the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, http://www.stratagene.com).

Stable transformation of wheat with *Pm3d+e*

Pm3d+e was amplified from the plasmid DNA construct used for the transient transformation assay by PCR using primers BamHI-1 (5'-TTAATTGGATCCCAATGGC-AGAGCGGGTGGTC-3') and Pm3-HA-stop-BamHI (5'-CATCATGGATCCTCAAGCAT-AATCTGGAACATCGTATGGATAGCTCCGGCAGGCCTGCCTCCG-3') and cloned into the *Bam*HI site of vector pAHC17 (Christensen and Quail 1996). The reverse primer introduces the HA (human influenza hemagglutinin) epitope tag encoding sequence at the 3' end of *Pm3d+e*. The gene cassette, consisting of the ubiquitin promoter, *Pm3d+e* and the nopaline synthase terminator, was released by restriction digest and co-transformed with the gene cassette of the selectable marker gene phosphomannose isomerase (Pmi, Reed *et al.* 2001). The transformation of immature embryos of hexaploid spring wheat cultivar Bobwhite SH 98 26 via particle bombardment and the regeneration of T_0 transformants was performed as described (Pellegrineschi *et al.* 2002, Wright *et al.* 2001).

Characterisation of transgenic *Pm3d+e* plants

Regenerated T_0 plants were screened by PCR using *Pm3*-specific primer pair SuB13 (5'-TGCCTAGAAGATCTATGCTTATCAG-3') and SuB8 (5'-CCGCTCACGGACTAGC-CTC-3'), which flank the *Pm3* intron sequence. Transgenic T_1 plants were analysed by DNA gel blots. Isolation of genomic DNA from leaves and Southern hybridisation were performed as described (Stein *et al.* 2001, Travella *et al.* 2006). DNA was digested with restriction enzymes *Bam*HI and *Dra*I. Membranes were hybridised with a 192-bp probe, *Pm3-intron*, which was amplified by PCR from the *Pm3d+e* plasmid construct using primers SuB15 (5'-CAGCACGTCCTTCTATC-3') and SuB16 (5'-ACTGCACATA-CCACAAG-3').

For powdery mildew infection tests, plants were raised in a growth chamber at 20°C/16°C day/night temperature with 70% relative humidity for ten days. Primary leaves of T_1 plants were cut into two segments of which one was infected with freshly propagated conidiospores (Winzeler *et al.* 1991) of powdery mildew isolate 97019, and the other with conidiospores of isolate DB Asosan. Leaf segments of T_2 plants were infected with isolates 97019, DB Asosan, 94202, 09003 and Ken 2-5. Scoring was performed as described by Kaur *et al*. (2008).

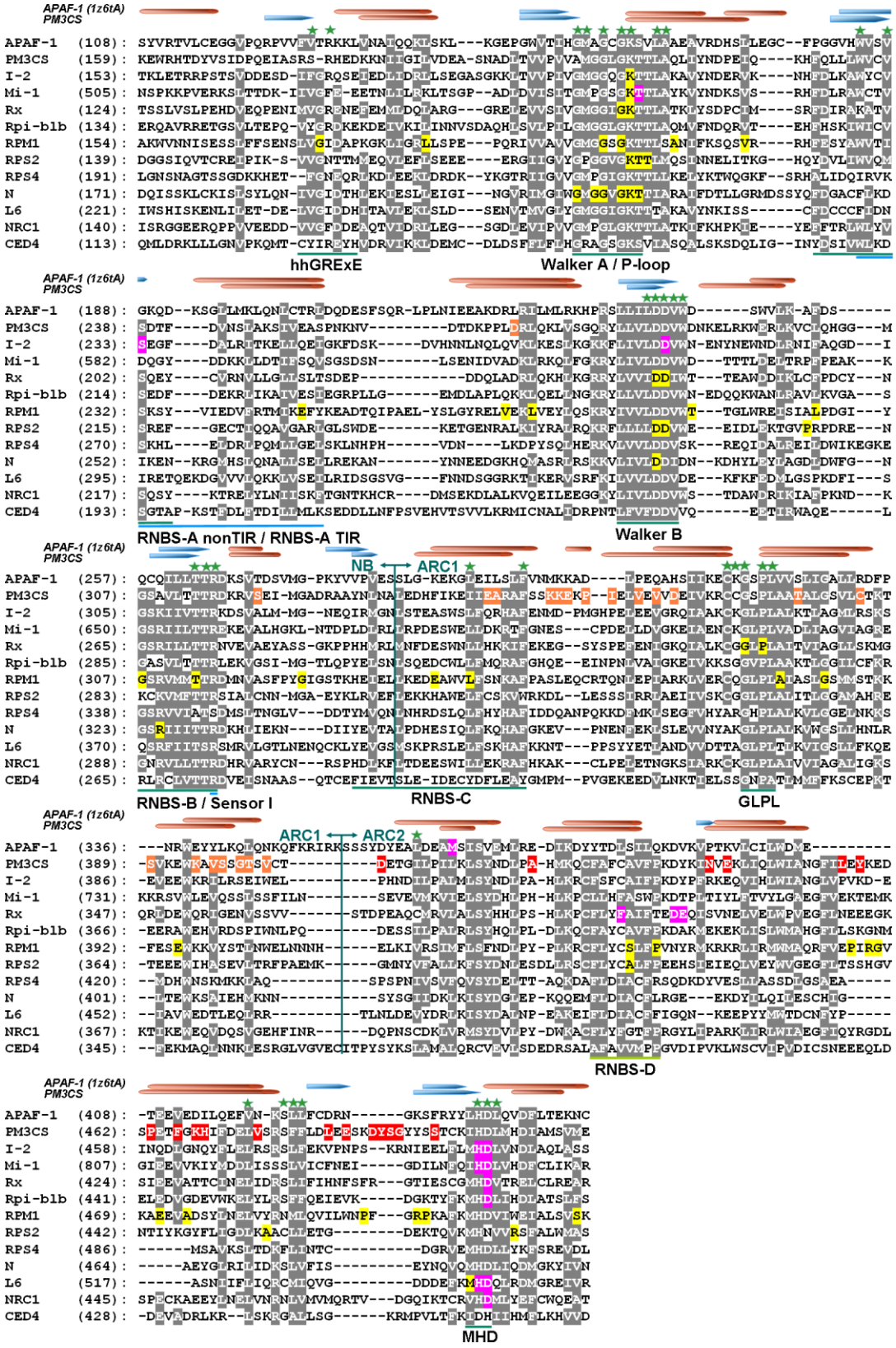

Figure S1. Structure-based multiple sequence alignment of the NB-ARC domain of PM3CS with APAF-1, CED-4 and ten plant R proteins.

The PM3CS sequence was added to the structure-based multiple sequence alignment published in van Ooijen *et al*. (2008b), including gain- and loss-of-function positions in pink and yellow, respectively. Green stars mark amino-acid positions that are conserved within R proteins and APAF-1 at the ADP binding site. Amino acids that are polymorphic in the corresponding PM3B sequence are coloured in orange; those that are polymorphic in both PM3A and PM3B are in red. The secondary-structure prediction reveals that PM3CS carries the vast majority of the α-helices (blue) and β-stands (brown) present in APAF-1. Furthermore, the alignment indicates that the NB-ARC domain encoded by *Pm3CS* contains all motifs of the three subdomains NB, ARC1 and ARC2. Only the first motif, hhGRExE, seems to be absent in PM3CS.


CC PM3CS MAERVVTMAIGPLVSMLKDKASSYLLDQYKVMEGMEEQHKILKRKLPAILDVITDVEEQAMAQREGAKAW
PM3CS LQELRTVAYVANEVFDEFKYEALRREAKKNGHYIKLGFDVIKLFPTHNRVAFRYKMGRKLCLILQAVEVL
PM3CS IAEMQVFGFKYQPQPPVS
Walker A/P-Loop
NB PM3CS KEWRHTDYVSIDPQEIASRSRHEDKKNIIGILVDEASNADLTVVPVVAMGGLGKTTLAQLIYNDPEIQKH
RNBS-A
Walker B/Kinase 2
NB PM3CS FQLLLWVCVSDTFDVNSLAKSIVEASPNKNVDTDKPPLDRLQKLVSGQRYLLVLDDVWDNKELRKWERLK
PM3B A
RNBS-B
NB PM3CS VCLQHGGMGSAVLTTTRDKRVSEIMGADRAAYNLNA
PM3B A
RNBS-C
GLPL
ARC1 PM3CS LEDHFIKEIIEARAFSSKKEKPIELVEVVDEIVKRCCGSPLAATALGSVLCTKTSVKEWKAVSSGTSVCT
PM3B VD ENG IP L M G S R T N IA RS I
RNBS-D
ARC2 PM3CS DETGILPILKLSYNDLPAHMKQCFAFCAVFPKDYKINVEKLIQLWIANGFILEYKEDSPETFGKHIFDEL
PM3A/B E S D A P H L I QL
MHD
NsiI
ARC2 PM3CS VSRSFFLDLEESKDYSGYYSS-TCKIHDLMHDIAMSVME
PM3A/B A I K EDWE RT
spacer PM3CS KECVVATMEPSEIEWLPDTARHLFLSCEEAERILNDSMQERSPAIQTLLCNSDVFSPLQHLSKYNT
PM3A/B T E D N K SS
PM3F E D N K SS
LRR1 PM3CS LHALKLCL-GTESFLLKPKYLHH
PM3A/B/F IR
PM3C R-
LRR2 PM3CS LRYLDLSESSIKALPEDISILYN
PM3A/F RM
PM3C Y E
LRR3 PM3CS LQVLDLSYCNYLDRLPRQMKYMTS
PM3C V N RS E
LRR4 PM3CS LCHLYTHGCRNLKSMPPGLENLTK
PM3C SK
PM3D/E W
LRR5 PM3CS LQTLTVFVAGVPGPDCADVGE
LRR6 PM3CS LHGLNIGGRLELCQVENVEKAEAEVANLGGQLE
BclI
LRR7 PM3CS LQHLNLGDQLELRRVENVKKAEAKVANLGNKKD
LRR8 PM3CS LRELTLRWTEVGDSKVLDKFEPHGG
LRR9 PM3CS LQVLKIYKYGGKCMGMLQN
LRR10 PM3CS MVEIHLSGCERLQVLFSCGTSFTFPK
LRR11 PM3CS LKVLTLEHLLDFERWWEINEAQEEQIIFPL
LRR12 PM3CS LEKLFIRHCGKLIALPEAPLLGEPSRGGNRLVCTPFSL
LRR13 PM3CS LENLFIWYCGKLVPLREAPLVHESCSGGYRLVQSAFPA
PM3A/F R N
LRR14 PM3CS LKVLALEDLGSFQKWDAAVEGEPILFPQ
PM3A/F E I
LRR15 PM3CS LETLSVQKCPKLVDLPEAPK
LRR16 PM3CS LSVLVIEDGKQEVFHFVDRYLSS
LRR17 PM3CS LTNLTLRLEHRETTSEAECTSIVPVDSKEKWNQKSP
LRR18 PM3CS LTVLELGCCNSFFGPGALEPWDYFVH
LRR19 PM3CS LEKLEIDRCDVLVHWPENVFQSLVS
PM3A K
LRR20 PM3CS LRTLLIRNCKNLTGYAQAPLEPLASERSQHPRG
PM3A R V E E L
AflII
LRR21 PM3CS LESLCLRNCPSLVEMFNVPAS
PM3A RIE
LRR22 PM3CS LKKMTIGGCIKLESIFGKQQGMAELVQVSSSSEAIMPATVSELPSTPMNHFCPC
PM3A D LE D
PM3D R
LRR23 PM3CS LEDLCLSACGSLPAVLNLPPS
LRR24 PM3CS LKTLEMDRCSSIQVLSCQLGGLQKPEATTSRSRSPIMPQPLAAATAPAAREHLLPPH
LRR25 PM3CS LEYLTILNCAGMLGGTLRLPAP
LRR26 PM3CS LKRLFIMGNSGLTSLECLSGEHPPS
PM3B/C I
LRR27 PM3CS LESLWLERCSTLASLPNEPQVYRS
PM3A/F KI D RS
PM3E V
PM3G Y D
LRR28 PM3CS LWSLEITGCPAIKKLPRCLQQQLGS
PM3D R
PM3G RY R
PM3CS IKRKWLDARYEVTEFKPLKPKTWKEIPRLVRERRQACRS

Figure S2. Amino acid differences of the PM3A – PM3G proteins.

Polymorphic amino acids are indicated below the PM3CS sequence, which is identical to the consensus sequence of the seven functional PM3 proteins. The protein domains are indicated on the left. The predicted CC structure is underlined. Motifs conserved in the NB-ARC domains of NBS-LRR proteins are underlined and labelled on top. The x-positions of the LxxLxLxx motif are highlighted in red, and the conserved leucines or other hydrophobic residues are represented in bold. Residues of the ARC2 subdomain that form loops in the 3D model (Figure 5) are coloured in green, and side chains of polymorphic residues that point to the solvent are highlighted in light blue. The notional positions of the *Afl*II, *Bcl*I and *Nsi*I restriction sites in the corresponding DNA sequence used for constructing chimeric genes are indicated.

Table S1. Numbers of powdery mildew isolates with the different (a)virulence gene combinations for *Pm3a/Pm3f* (a) and *Pm3b/Pm3c* (b).

(a)

	AvrPm3a	*avrPm3a*
AvrPm3f	173	0
avrPm3f	281	40

(b)

	AvrPm3b	*avrPm3b*
AvrPm3c	337	0
avrPm3c	317	56

Table S2. *P* values of Student's *t*-test on HI of constructs shown in Figure 3.

Isolate 97028

	Pm3a	Pm3f	Pm3a-f_{ARC}	Pm3f-a_{ARC}	Pm3a-$f_{LRR19-22}$
Pm3CS	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Pm3a	-	< 0.001	< 0.001	0.003	0.001
Pm3f	-	-	< 0.001	< 0.001	< 0.001
Pm3a-f_{ARC}	-	-	-	0.031	0.053
Pm3f-a_{ARC}	-	-	-	-	0.116

Isolate 96224

	Pm3a	Pm3f	Pm3a-f_{ARC}	Pm3f-a_{ARC}	Pm3a-$f_{LRR19-22}$
Pm3CS	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Pm3a	-	0.747	0.661	0.576	0.519
Pm3f	-	-	0.427	0.790	0.776
Pm3a-f_{ARC}	-	-	-	0.310	0.212
Pm3f-a_{ARC}	-	-	-	-	0.939

Isolate 07201

	Pm3a-f_{ARC}	Pm3f-a_{ARC}	Pm3a-$f_{LRR19-22}$
Pm3CS	0.981	0.568	0.068
Pm3a-f_{ARC}	-	0.764	0.314
Pm3f-a_{ARC}	-	-	0.096

Table S3. *P* values of Student's *t*-test on HI of constructs shown in Figure 4a.

Isolate 97011

	Pm3d	Pm3b-d_{NB-ARC}	Pm3d-b_{NB-ARC}
Pm3b	0.002	0.523	0.002
Pm3d	-	< 0.001	0.046
Pm3b-d_{NB-ARC}	-	-	< 0.001

Isolate 96229

	Pm3d	Pm3b-d_{NB-ARC}	Pm3d-b_{NB-ARC}
Pm3b	< 0.001	0.002	< 0.001
Pm3d	-	0.002	0.004
Pm3b-d_{NB-ARC}	-	-	0.051

Table S4. *P* values of Student's *t*-test on HI of constructs shown in Figures 4a and 6a (virulent controls).

Isolate		Pm3d-b_{NB-ARC}	Pm3b-CS_{LRR26}	Pm3c-CS_{LRR26}
07016	Pm3CS	0.341	0.918	-
07296	Pm3CS	-	-	0.238

Table S5. *P* values of Student's *t*-test on HI of constructs shown in Figure 4b.

Isolate 96229

	Pm3b	Pm3CS-b_{NB-ARC}
Pm3CS	< 0.001	0.817
Pm3b	-	< 0.001

Isolate 96224

	Pm3b	Pm3CS-b_{NB-ARC}
Pm3CS	< 0.001	0.983
Pm3b	-	< 0.001

Table S6. *P* values of Student's *t*-test on HI of constructs shown in Figure 6a.

Isolate 96224

	Pm3b	Pm3c	Pm3b-CS_{LRR26}	Pm3c-CS_{LRR26}	Pm3CS-b/c_{LRR26}
Pm3CS	< 0.001	< 0.001	< 0.001	< 0.001	0.002
Pm3b	-	0.927	< 0.001	< 0.001	0.001
Pm3c	-	-	< 0.001	< 0.001	< 0.001
Pm3b-CS_{LRR26}	-	-	-	0.960	0.122
Pm3c-CS_{LRR26}	-	-	-	-	0.120

Isolate 97019

	Pm3c	Pm3c-CS_{LRR26}	Pm3CS-b/c_{LRR26}
Pm3CS	< 0.001	0.004	0.905
Pm3c	-	< 0.001	< 0.001
Pm3c-CS_{LRR26}	-	-	0.003

Table S7. *P* values of Student's *t*-test on HI of constructs shown in Figure 6b.

Isolate 97011

	Pm3d	Pm3b-$d_{LRR22,28}$	Pm3d-b/c_{LRR26}
Pm3b	0.002	0.923	0.313
Pm3d	-	< 0.001	< 0.001
Pm3b-$d_{LRR22,28}$	-	-	0.111

Isolate 96229

	Pm3d	Pm3b-$d_{LRR22,28}$	Pm3d-b/c_{LRR26}
Pm3b	< 0.001	< 0.001	< 0.001
Pm3d	-	0.001	0.172
Pm3b-$d_{LRR22,28}$	-	-	< 0.001

Table S8. *P* values of Student's *t*-test on HI of constructs shown in Figure 7.

Isolate 97019

	Pm3e	Pm3d+e
Pm3d	< 0.001	< 0.001
Pm3e	-	0.401

Isolate DB Asosan

	Pm3d	Pm3e	Pm3d+e
Pm3CS	< 0.001	0.005	< 0.001
Pm3d	-	< 0.001	0.615
Pm3e	-	-	< 0.001

Isolate 94202

	Pm3d	Pm3e	Pm3d+e
Pm3CS	0.364	< 0.001	< 0.001
Pm3d	-	< 0.001	< 0.001
Pm3e	-	-	0.087

3. Rye *Pm8* and wheat *Pm3* are orthologous genes and show evolutionary conservation of resistance function against powdery mildew

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3.1 Summary

The improvement of wheat through breeding has relied strongly on the use of genetic material from related wild and domesticated grass species. The 1RS chromosome arm from rye was introgressed into wheat and crossed into many wheat lines, as it improves yield and fungal disease resistance. *Pm8* is a powdery mildew resistance gene on 1RS which, after widespread agricultural cultivation, is now widely overcome by adapted mildew races. Here we show by homology-based cloning and subsequent physical and genetic mapping that *Pm8* is the rye orthologue of the *Pm3* allelic series of mildew resistance genes in wheat. The cloned gene was functionally validated as *Pm8* by transient, single-cell expression analysis and stable transformation. Sequence analysis revealed a complex mosaic of ancient haplotypes among *Pm3*- and *Pm8*-like genes from different members of the Triticeae. These results show that the two genes have evolved independently after the divergence of the species 7.5 million years ago and kept their function in mildew resistance. During this long time span the co-evolving pathogens have not overcome these genes, which is in strong contrast to the breakdown of *Pm8* resistance since its introduction into commercial wheat 70 years ago. Sequence comparison revealed that evolutionary pressure acted on the same subdomains and sequence features of the two orthologous genes. This suggests that they recognize directly or indirectly the same pathogen effectors that have been conserved in the powdery mildews of wheat and rye.

3.2 Introduction

In the agricultural environment, plants are under permanent attack from pathogens. Resistance breeding in crop species strongly relies on resistance (*R*) genes. They encode proteins which directly or indirectly recognize effector molecules delivered into plant cells by pathogens (Dodds and Rathjen 2010). In the co-evolutionary arms race of host and pathogen, the emergence of an effector gene leading to successful pathogen invasion is followed by the emergence of a new *R* gene in the host plant (Kanzaki *et al.* 2012). *R* genes frequently break down rapidly because there is strong selection for virulent pathogen mutants under agricultural conditions (McDonald and Linde 2002). Therefore, resistance breeding depends on the constant identification of new resistance resources to be integrated into breeding programmes.

The elite wheat (*Triticum aestivum* L.) cultivars currently grown agriculturally only represent a small fraction of the wheat gene pool. In order to further improve wheat for resistance to biotic and abiotic stresses, quality and yield, breeders have been using the genetic diversity of closely related wild and domesticated species (Baum *et al.* 1992). The transfer of alien chromatin to wheat resulted in agronomically useful wheat-alien translocation lines that carry important functional resistance genes against several pathogens, mainly rust and powdery mildew (Friebe *et al.* 1996, Tyrka and Chelkowski 2004). Despite their agronomical importance, little is known about these translocations on the molecular level and it remains to be determined whether single genes or gene clusters mediate the observed resistance traits.

In the 1930s, a wheat cultivar was developed which carried the translocated 1RS chromosome arm of the rye (*Secale cereale* L.) cultivar Petkus, replacing wheat chromosome arm 1BS. Cultivars with this so-called 1BL.1RS translocation (Mettin *et al.* 1973, Zeller 1973) showed high yield potential, wide adaptation and disease resistance

against powdery mildew, stem rust, leaf rust and stripe rust (Kim *et al.* 2004, Rabinovich 1998). Due to these favourable traits, in 1998 wheat-rye translocation lines accounted for 50% of the International Maize and Wheat Improvement Center (CIMMYT) high-yielding bread wheat cultivars, covered over 5 million hectares of the wheat grown area worldwide and are still cultivated at large scale (Purnhauser *et al.* 2011, Villareal *et al.* 1998). The gene on chromosome arm 1RS conferring resistance to wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*) was named *Pm8* (McIntosh 1988). Soon after its widespread use in the 1970s, increasing mildew virulence to *Pm8* was reported (Bennett 1984, Heun and Friebe 1990).

To date, in the wheat gene pool 43 genetic loci with nearly 70 genes/alleles have been described to mediate resistance against wheat powdery mildew. More than 30 of these *Pm* genes/alleles were introgressed from wild relatives, demonstrating the widespread use of resistance genes from foreign species in wheat breeding (McIntosh *et al.* 2012, Tyrka and Chelkowski 2004). So far, only two *Pm* genes have been cloned: *Pm3* from wheat (Yahiaoui *et al.* 2004) and a key member of the *Pm21* resistance locus transferred from *Haynaldia villosa* to hexaploid wheat (Cao *et al.* 2011). For *Pm3*, 17 functional alleles were isolated which share more than 97% nucleotide sequence identity and code for coiled-coil (CC), nucleotide-binding site, ARC1 and ARC2 (NB-ARC) and leucine-rich-repeat (LRR) domain proteins (Bhullar *et al.* 2009, Bhullar *et al.* 2010, Srichumpa *et al.* 2005, Yahiaoui *et al.* 2006, Yahiaoui *et al.* 2009, Yahiaoui *et al.* 2004).

The last common ancestor of wheat and rye lived around 7.5 million years ago (Huang *et al.* 2002a, Huang *et al.* 2002b). Both wheat and rye are hosts of the cereal powdery mildews (*Blumeria graminis*) but due to strict host specialization they are infected by two different *formae speciales*, f.sp. *tritici* (*Bgt*) and f.sp. *secalis* (*Bgs*), respectively. From barley (f.sp. *hordei*) and wheat powdery mildew it is known that the hosts

separated about 12 million years ago while the pathogen separated about 2 million years later, indicating host-pathogen co-evolution (Oberhaensli *et al.* 2011). This might also be true for wheat and rye powdery mildew. Analysis of individuals of a cross between wheat and rye powdery mildew demonstrated that avirulence genes from the rye powdery mildew are recognized by wheat *R* genes active against wheat powdery mildew (Matsumura and Tosa 1995). This suggests that *R* genes might be involved in non-host resistance to inappropriate *formae speciales*. A model was proposed in which both NB-LRR genes and pattern recognition receptors (PRRs) mediate nonhost resistance and their relative contribution depends on the divergence time between the host and the nonhost plant (Schulze-Lefert and Panstruga 2011).

Resistance genes are generally assumed to be rapidly evolving (Michelmore and Meyers 1998) and functional prediction of *R* gene homologues/orthologues in related species has been shown to be unreliable (Hulbert *et al.* 2001). Resistance function against different pathogens has been shown for the homologous pairs *Tm-2*^{2}/*Rpi-vnt1.1* and *Rpi-blb2*/*Mi-1* from *Solanum* species (Foster *et al.* 2009, van der Vossen *et al.* 2005) as well as for the three allelic *Arabidopsis* genes *RPP8*, *HRT* and *RCY1* (Takahashi *et al.* 2002). Recently, the stem rust resistance gene *Sr33* from *Aegilops tauschii* was found to be a homologue of the *Mla* resistance gene family in barley which mediates resistance to powdery mildew (Periyannan *et al.* 2013). In contrast, recognition of the same pathogen was shown for the homologous genes *N´* from *Nicotiana sylvestris* and *L* from *Capsicum* against *Tobamovirus* spp. in a transient expression assay in *Nicotiana benthamiana* (Sekine *et al.* 2012) and three orthologous *R* genes at the *Xa3*/*Xa26* locus in one cultivated and two wild rice cultivars against *Xanthomonas oryzae* (Li *et al.* 2012). An example of conserved fungal pathogen recognition from grass species comes from *TmMla1* isolated from the wheat species *Triticum monococcum* mediating powdery mildew resistance like its barley homolog

Mla (Jordan *et al.* 2011). These examples of *R* gene homologues gave first insights into the evolution of *R* genes. However, the orthologous relationship remains unclear for most of the genes and little is known about the evolution of true orthologous *R* genes mediating resistance to the same pathogen.

In this study, we cloned the *Pm8* gene by a homology-based cloning approach and found it to be the rye orthologue of the wheat *Pm3* gene. Sequence analysis revealed that the PM8 and PM3B proteins share 81% sequence identity and that nucleotide diversity is located mainly in the solvent-exposed residues of the LRR domain. The data indicate that an orthologous gene in two grass species can co-evolve with related pathogens over a surprisingly long evolutionary time.

3.3 Results

3.3.1 Homology-based cloning of a candidate gene for *Pm8*

Pm8 maps to a gene-dense region at the distal end of chromosome arm 1RS (Hulbert *et al.* 2001, Sandhu and Gill 2002), whereas the wheat *Pm3* powdery mildew resistance gene is located in the syntenic gene-dense region of chromosome arm 1AS of wheat, close to the genetic loci encoding the storage proteins *Glu-3*/*Gli-1*. Considering that gene order is highly conserved in grasses (Elliott *et al.* 2002, Mohler *et al.* 2002, Sandhu and Gill 2002), we hypothesized that the two genes are orthologues.

To determine whether *Pm3* and *Pm8* show any similarity in their powdery mildew resistance spectra, we analysed the resistance spectra of *Pm8* and different *Pm3* alleles to 162 wheat powdery mildew isolates from Switzerland (Brunner *et al.* 2011), seven from USA and 24 from France (Methods S1). The isolates were tested on wheat differential lines for *Pm8* and the *Pm3a-g* alleles, except for 33 Swiss isolates for which *Pm3e* and *Pm3g* data were missing. The 35 isolates found to be avirulent on *Pm8* were all also avirulent to at least one of the tested *Pm3* alleles. The 13 isolates which were virulent on all tested *Pm3* alleles were also virulent on *Pm8*. The same correlations were also detected in other virulence analyses on wheat powdery mildew isolates from Germany and England (Huang and Röder 2004, Lillemo *et al.* 2010, Schmolke *et al.* 2012, Zeller *et al.* 2002). This functional similarity between *Pm8* and the *Pm3* alleles possibly reflects recognition of similar effector proteins and provided additional support for the hypothesized orthologous relationship between the two genes.

Southern blot analysis was carried out to examine the presence of *Pm3* homologous genes on the rye chromosome arm 1RS from ‘Petkus’ carrying the powdery mildew resistance gene *Pm8*. A 184-bp fragment from the wheat cultivar Chul/8*Chancellor

located 4kb upstream of the *Pm3b* allele was used as a probe. This probe UP3 (Figure 1a) is a fragment of the restriction fragment length polymorphism probe *TmRGL-1pro* which has been shown to specifically detect *Pm3* loci in hexaploid wheat (Srichumpa *et al.* 2005, Yahiaoui *et al.* 2004), *T. monococcum* and *Triticum turgidum* (Wicker *et al.* 2007a). It does not hybridize to closely related members of the large *Pm3* resistance gene-like (RGL) family in these genomes. Remarkably, all tested 1BL.1RS wheat lines and the three 'Petkus' rye lines Petkuser Winter, Petkus II and Petkus 91 shared a distinct fragment of 4 kb (Figure 1b). This fragment is absent in Chul/8*Chancellor and the rye cultivar Imperial, two lines not carrying *Pm8*. In the wheat line Kavkaz, an additional fragment of 6.5 kb indicates the presence of the *Pm3* allele *Pm3-Kavkaz* on wheat chromosome 1AS (Yahiaoui *et al.* 2006). Thus, Southern blot analysis indicated the presence of one *Pm3* homologue on the chromosome arm 1 RS in lines carrying *Pm8*, and this sequence represented a good candidate for *Pm8*.

We then used available sequence information of the known *Pm3* alleles for a homology-based approach to clone this candidate gene. First, the 5' untranslated region and the 5' end of the candidate gene were amplified using the primers UP3B/consLRR3B2 (Figure 1a). The 3' end of the gene was amplified by 3' rapid amplification of cDNA ends (RACE)-PCR (Methods S1 in Supporting Information). Based on the obtained 5' and 3' sequences, the full-length open reading frame was amplified from wheat line Kavkaz/4*Federation and rye line Petkus 91 genomic DNA using nested PCR similar to the amplification strategy for the *Pm3* alleles (Srichumpa *et al.* 2005). The sequences amplified from the wheat translocation line and from rye were identical. The predicted gene has a total length of 4321 bp, with one 193-bp intron ending 84 bp upstream of the stop codon, as deduced from the 3' RACE sequence information. Based on the successful amplification in the 3' RACE experiment we

conclude that the gene is expressed. It encodes a protein of 1,375 amino acids and we refer to it as the *Pm8*-candidate gene.

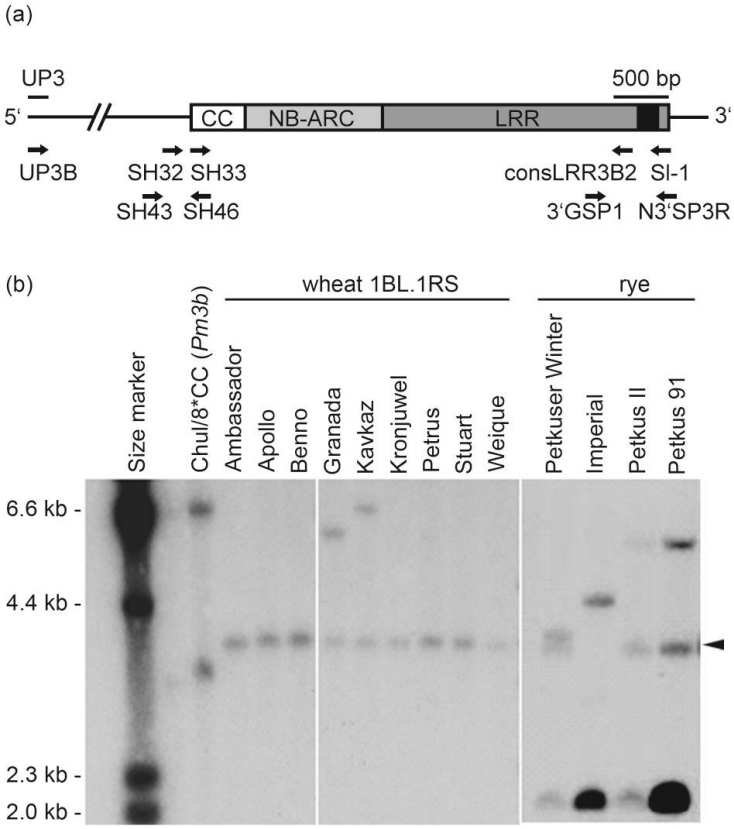


Figure 1. Wheat lines carrying the 1BL.1RS chromosome and three 'Petkus' rye lines share a *Pm3* homologous sequence.

(a) The structure of the *Pm8*-candidate gene is shown. The black box indicates the intron and the other boxes the coding region with its three predicted domains [coiled coil (CC), nucleotide-binding (NB)-ARC, leucine-rich repeat (LRR)]. The 2.7-kb non-coding 5' sequence including the promoter region and the 0.4 kb untranslated 3' end are represented as lines. The primers used for cloning of the *Pm8*-candidate gene are indicated by arrows. The wheat probe UP3 was used for Southern blot analysis (its homologous sequence in the *Pm8*-candidate gene is indicated by a horizontal bar).

(b) Wheat and rye genomic DNA was digested with *Hin*dIII and hybridized with the 184-bp UP3 probe. A fragment of 4 kb was detected in all tested wheat 1BL.1RS and 'Petkus' rye lines (indicated by the arrow head). Line Chul/8*Chancellor (Chul/8*CC) contains the *Pm3b* allele and was used as a positive control.

3.3.2 The *Pm8*-candidate gene maps to the *Pm8* locus

The sequence of the *Pm8*-candidate gene, including its 5' and 3' regions, was compared with all available sequences from wheat with homology to the *Pm3* alleles (Bhullar *et al.* 2010, Wicker *et al.* 2007a, Yahiaoui *et al.* 2004). This allowed us to design a primer pair specific for the *Pm8*-candidate gene (SH43/SH46; Figure 1a), which amplified a 662-bp fragment in wheat and rye lines carrying *Pm8* (Figure 2). Validation of this marker *sfr43(Pm8)* on a large set of wheat lines with and without the 1BL.1RS translocation as well as on rye lines proved it to be diagnostic for the presence of the *Pm8* gene (Table S1).

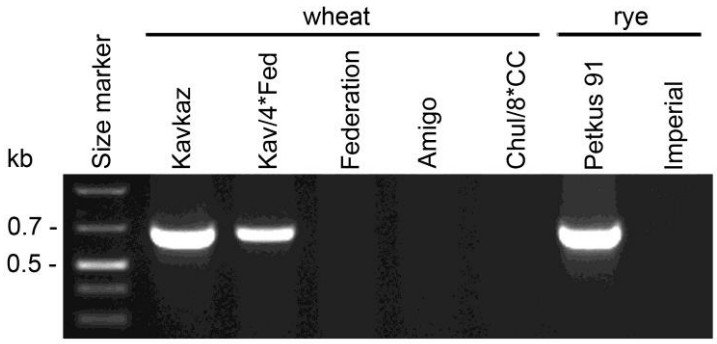


Figure 2. The *Pm8*-candidate gene marker *sfr43(Pm8)*.
The primer pair SH43/SH46 amplifies a 662-bp fragment in wheat 1BL.1RS lines (Kavkaz and Kavkaz/4*Federation) as well as in rye line Petkus 91. No fragment was amplified from wheat lines without the 1BL.1RS translocation [Federation, Amigo (1AL.1RS; *Pm17*) and Chul/8*Chancellor (*Pm3b*)] or from rye line Imperial known not to carry *Pm8*.

We used two independent populations to genetically and physically map the *Pm8*-candidate gene. In an earlier study of wheat 1BS/1RS homoeologous recombinants obtained in a *ph1b* mutant background, the *Pm8* resistance phenotype was shown to be genetically located between the cluster of rust resistance genes *Lr26*, *Sr31* and *Yr9* and the *Gli-1*/*Glu-3* loci (Lukaszewski 2000, Sharma *et al.* 2009). To test if the *Pm8*-candidate gene maps to this same locus, we analysed six recombinants with physical breakpoints close to *Pm8* with marker *sfr43(Pm8)*, along with their parental lines Pavon (no *Pm8*) and Pavon 1RS.1BL (*Pm8*). The *Pm8* resistance phenotype was confirmed in the recombinants using a *Pm8* avirulent powdery mildew isolate, 07230, in a leaf segment infection test (Methods S1). The recombinants T9 and 1B+37 were resistant in the infection test and were positive for the marker *sfr43(Pm8)*. The other four recombinants (T16, T18, T8 and 1B+14) were susceptible in the infection test and negative for the marker (Figure 3a). These results are consistent with previous infection tests and showed that the *Pm8*-candidate gene co-localized with the *Pm8*-mediated powdery mildew resistance and with the physical map position of the *Pm8*-locus.

In a second approach, we used a high-resolution mapping population from a cross of two 1BL.1RS wheat lines earlier developed for the rust resistance genes *Sr31*, *Lr26* and *Yr9* on chromosome arm 1RS (Mago *et al.* 2005). This population was segregating for *Pm8* but it was not phenotyped for this gene. Since no more seeds of the F_2 population were available, only genomic DNA, no phenotypic analysis could be performed. In total, 134 F_2 plants were screened with the marker *sfr43(Pm8)*. It mapped 0.7 cM proximal to the marker *Xiag95* and 1.7 cM distal to the rust resistance genes (Figure 3b). The location of *Pm8* in this mapping population is in agreement with the location of *Pm8* found in the 1BS/1RS homoeologous recombinants, where *Pm8* is located between markers *Gli-1*/*Glu-3* (and Iag95; Mago *et al.* 2002) and the rust resistance genes (Figure 3a).

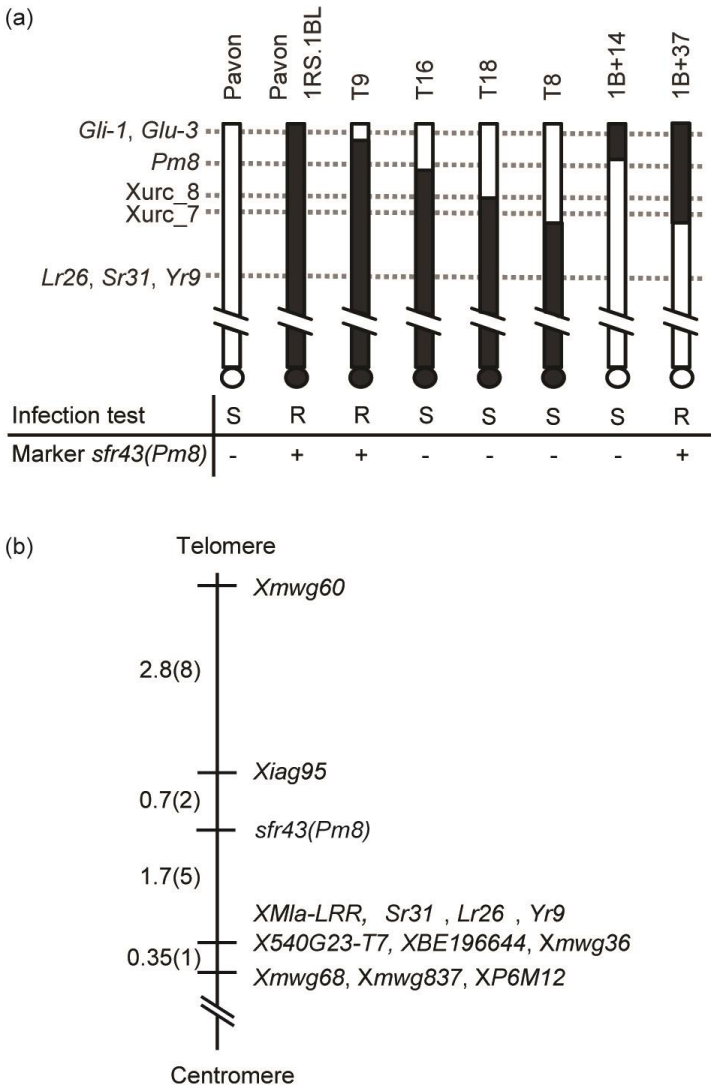


Figure 3. The *Pm8*-candidate gene marker *sfr43(Pm8)* maps to the *Pm8* resistance locus.

(a) Molecular analysis of wheat-rye recombinants. Black bars indicate the rye chromosome arm 1RS and white bars the wheat chromosome arm 1BS. On the left side, markers and gene locations are indicated (Sharma *et al.* 2009). Below the graph, results from the infection test with powdery mildew isolate 07230 (R, resistant; S, susceptible) and results of the *Pm8*-candidate gene marker *sfr43(Pm8)* analysis are given (+, 662-bp fragment present; -, no fragment).

(b) The *Pm8*-candidate gene marker *sfr43(Pm8)* was integrated into the genetic map established by Mago *et al*. (2005) by testing 134 F_2 wheat individuals. On the left, distances between markers are given in cM and the number of recombinants is indicated in brackets.

3.3.3 The *Pm8*-candidate gene mediates race-specific resistance to powdery mildew

We validated the *Pm8*-candidate gene for resistance function by transiently expressing it in leaf epidermal cells of the wheat cultivars Federation and Chancellor, which are highly susceptible to powdery mildew. Transient expression of the *Pm8*-candidate gene resulted in a significant reduction of the haustorium index (HI) (HI 27% Federation and 26% Chancellor; Student's *t*-test, $P < 0.001$) compared with the empty vector control (HI 66% Federation and 67% Chancellor) when the leaves were inoculated with the *Pm8* avirulent powdery mildew isolate 07230 (Figure 4a, Table S2). In contrast, there was no significant difference in the HI when a *Pm8* virulent isolate 07250 was used in this assay (HI 62% Federation and 67% Chancellor; Student's *t*-test, $P > 0.7$) (Figure 4a, Table S2). This demonstrated that the *Pm8*-candidate gene mediated race-specific powdery mildew resistance to an isolate avirulent on *Pm8*.

We then stably transformed the *Pm8*-candidate gene under the transcriptional control of the maize ubiquitin promoter (*ubi*) into cultivar Bobwhite SH 98 26, which is highly susceptible to powdery mildew and does not carry an endogenous *Pm8* gene. Functionality of this construct was first confirmed in transient transformation assays. Three transformation events, which segregated for powdery mildew resistance in the T_1 generation and carried one to three copies of the gene, as indicated by Southern blot analysis (Figure S1), were further analysed. In the T_2 generation, the heterozygous families showed co-segregation of the transgene with *Pm8* powdery mildew resistance, while plants of the homozygous families (T_2-Pm8#12, T_2-Pm8#34 and T_2-Pm8#59) were resistant when inoculated with an isolate which is avirulent on *Pm8* (07230). All plants were susceptible to *Pm8*-virulent isolate 07250 (Figure 4b).

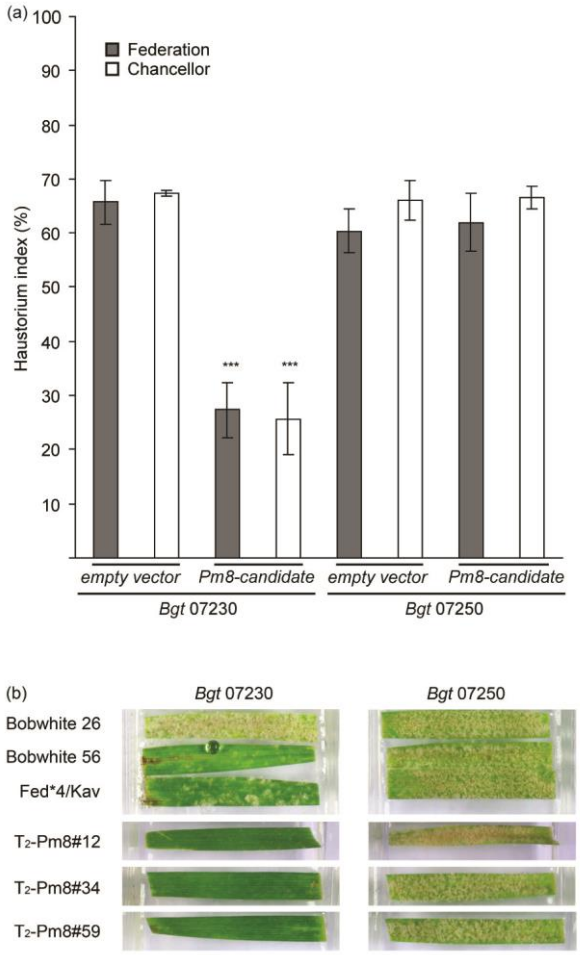


Figure 4. The *Pm8*-candidate gene mediates race-specific powdery mildew resistance against isolate *Bgt* 07230.

(a) In the transient assay, the haustorium index (HI) was significantly lower when leaf segments of the susceptible wheat cultivars Federation or Chancellor were bombarded with the *Pm8*-candidate gene and infected with the *Pm8* avirulent *Bgt* isolate 07230 compared with the empty vector control (Student's *t*-test, ***$P < 0.001$). No significant difference was found when using virulent *Bgt* isolate 07250 (Student's *t*-test, $P > 0.7$). The HIs represent the mean of three independent biological replicates and error bars are the standard deviations.

(b) The wheat cultivar Bobwhite 26 (Bobwhite SH 98 26) was stably transformed with the *Pm8*-candidate gene. While Bobwhite 26 was highly susceptible to the isolates 07230 and 07250, T_2 plants of three independent transformation events (Pm8#12, Pm8#34 and Pm8#59) were completely resistant to isolate 07230 but susceptible to isolate 07250. The control cultivars Bobwhite 56 (Bobwhite SH 98 56) and Fed*4/Kav (Federation*4/Kavkaz) carry an endogenous *Pm8* gene and are resistant to isolate 07230 but susceptible to isolate 07250.

Expression of the *Pm8* transgene was verified in a reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) assay along with the wheat-rye translocation lines Ambassador, Benno, Federation*4/Kavkaz and Veery#6 and the rye line Petkus 91, all carrying an endogenous *Pm8* gene. The *Pm8* expression levels in these control lines were not significantly different from each other. In contrast, transgenic line Pm8#12 showed an expression level approximately 430 times higher than the Federation*4/Kavkaz line, while lines Pm8#34 and Pm8#59 had expression levels about 255 and 161 times higher, respectively (Figure 5). The complete resistance of the transgenic lines to the *Pm8*-avirulent isolate compared with the incomplete resistance of the endogenous *Pm8* line Federation*4/Kavkaz (Figure 4b) could be due to the high expression level of *Pm8* under the ubiquitin promoter. The genetic background may also play a role, since a related Bobwhite line carrying an endogenous *Pm8* gene (Bobwhite SH 98 56) was more resistant than Federation*4/Kavkaz (Figure 4b). In summary, the *Pm8*-candidate gene-mediated race-specific powdery mildew resistance is identical to the resistance mediated by the endogenous *Pm8* gene both in a transient assay and in stably transformed lines. The functionality of the *Pm8*-candidate gene in three distinct wheat cultivars in the absence of rye chromosome 1RS indicates that *Pm8* function is cultivar independent and does not rely on additional genes present on the rye 1RS translocation. We conclude that the isolated gene is *Pm8*.

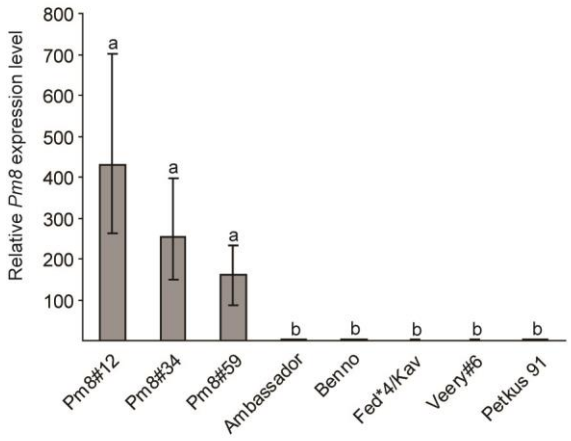


Figure 5. The transgenic *Pm8* lines showed a higher expression level of *Pm8* than the wheat-rye translocation lines and rye.

The relative expression levels of *Pm8* were determined by reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) in the first leaf of 12-day-old plants. They are plotted relative to line Fed*4/Kav (Federation*4/Kavkaz) and represent the means (untransformed) of three biological replicates (two replicates for Petkus 91). The 95% confidence intervals (back-transformed) are plotted. On top of the bars, different letters denote a significant difference in expression level (Tukey's honestly significant difference test, α = 0.050).

3.3.4 *Pm3*-specific haplotype sequence at the *Pm8* locus reveals orthology

A BLAST similarity search revealed that *Pm8* is most similar to the *Pm3* alleles showing 87% identity at the DNA level and 80% at the protein level to the *Pm3* allele *Pm3CS*. *Pm3CS* is a susceptible allele present in several wheat lines and represents the consensus sequence of the known wheat *Pm3* resistance alleles (Yahiaoui *et al.* 2006). The highest similarity was found to the functional allele *Pm3b* with 81% identity at the protein level. The *Pm8* gene codes for a protein of 1375 amino acids, whereas *Pm3b* codes for 1415 amino acids. The intron size is highly similar with 193 bp for *Pm8* versus 200 bp for *Pm3b* and is conserved in its position at the C-terminal end of the protein. All of the characteristic domains of coiled-coil NB-LRR resistance proteins as

well as all the motifs in the NB-ARC domain (Takken *et al.* 2006) are conserved in length and position between the *Pm3* alleles and *Pm8* (Figure S2).

The UP3 Southern blot probe was shown in earlier studies to specifically detect *Pm3* among a large number of sequence-related genes in wheat (Yahiaoui *et al.* 2004). A BLASTN search including the whole wheat survey sequences (http://www.wheatgenome.org/) revealed that the probe had no other homology than to the 5' region of the *Pm3* alleles, a bacterial artificial chromosome clone from *T. monococcum* which was used for mapping of the *Pm3* locus and a *Pm3* homologue on chromosome 1BS (*Pm3*-1B). In the barley genome (Mayer *et al.* 2012) the sequence is not conserved, while in rye three copies are present as indicated from Southern blot analysis (Figure 1b). Sequence comparison of *Pm8* and *Pm3* showed that UP3 is part of a 294-bp region that is highly conserved between the two genes (94% identity; Figure S3). This is even more remarkable considering that the complete 5' regions of the two genes are in general highly diverse with 54% identity (Figure S3). The fact that the *Pm3* haplotype-specific sequence is conserved in *Pm8* strongly supports the conclusion from physical and genetic mapping experiments that *Pm8* and *Pm3* are orthologues.

3.3.5 Very high sequence conservation in the CC domain between *Pm8* and *Pm3*

Among the CC, NB-ARC and LRR domains, the CC domain shows the highest sequence conservation, with only 10 out of 158 amino acids being different between PM8 and PM3B in this domain (Figure S2). This is reflected by a very low nucleotide diversity value of 0.051 (π(total); Table 1), while the nucleotide diversity value of *Pm8* compared with *Pm3b* for the entire gene is 0.097. Actually, in the predicted CC structure only one amino acid change is found. Interestingly, no polymorphisms were found in the CC domain among the 31 *Pm3* alleles known from hexaploid wheat, and

only three nucleotide polymorphisms were found in the 23 alleles from tetraploid wheat (Bhullar *et al.* 2010, Yahiaoui *et al.* 2009). The CC domain of NB-LRR R proteins has been shown to specifically interact with other proteins or to form oligomers (Chang *et al.* 2013, Jordan *et al.* 2011, Maekawa *et al.* 2011a, Rairdan *et al.* 2008, Shen *et al.* 2007). We speculate that the CC domains of PM3 and PM8 may interact with the same proteins and are involved in the same signalling pathway based on their high sequence conservation.

Table 1. Nucleotide diversity between *Pm8* and *Pm3b*.

Structure	Number of sites	Polymorphic sites	Synonymous changes	Non-synonymous changes	π (total)[a]	π syn[a]	π ns[a]
Entire gene	4122	400	146	253	0.097	0.14	0.084
CC domain	474	24	14	10	0.051	0.124	0.029
NB domain	519	78	31	46	0.15	0.245	0.121
ARC1 domain	210	30	6	24	0.143	0.082	0.161
ARC2 domain	327	5	4	1	0.015	0.053	0.006
SPA	195	32	11	21	0.164	0.24	0.142
LRR domain	2397	231	80	151	0.096	0.129	0.087
Solvent-exposed residues	402	-	16	46	0.096	0.143	0.157

CC, coiled coil; NB, nucleotide-binding; LRR, leucine-rich repeat.
[a] π is the nucleotide diversity which gives the average number of nucleotide differences per site between sequences. syn,synonymous; ns, non-synonymous

3.3.6 Sequence comparison of the LRR domains

It was shown previously that LRR6 and LRR7 in the LRR domain encoded by the *Pm3* alleles are nearly identical in sequence and therefore result from a duplication event (Wicker *et al.* 2007b, Yahiaoui *et al.* 2004). This LRR duplication was also found to be present in a *Pm3*-like gene isolated from the D genome progenitor of wheat, *Ae. tauschii* (Figures 6 and S2). However, it did not occur in several other *Pm3*-like genes from *T. aestivum*, *T. monococcum*, *T. turgidum*, barley and rice (Wicker *et al.* 2007b), nor in *Pm8* (Figure S2). Therefore, this duplication event must have occurred after the speciation of wheat and rye about 7.5 million years ago and most likely before the three wheat diploid ancestor genomes separated about 2.5 million years ago (Chalupska *et al.* 2008, Huang *et al.* 2002a).

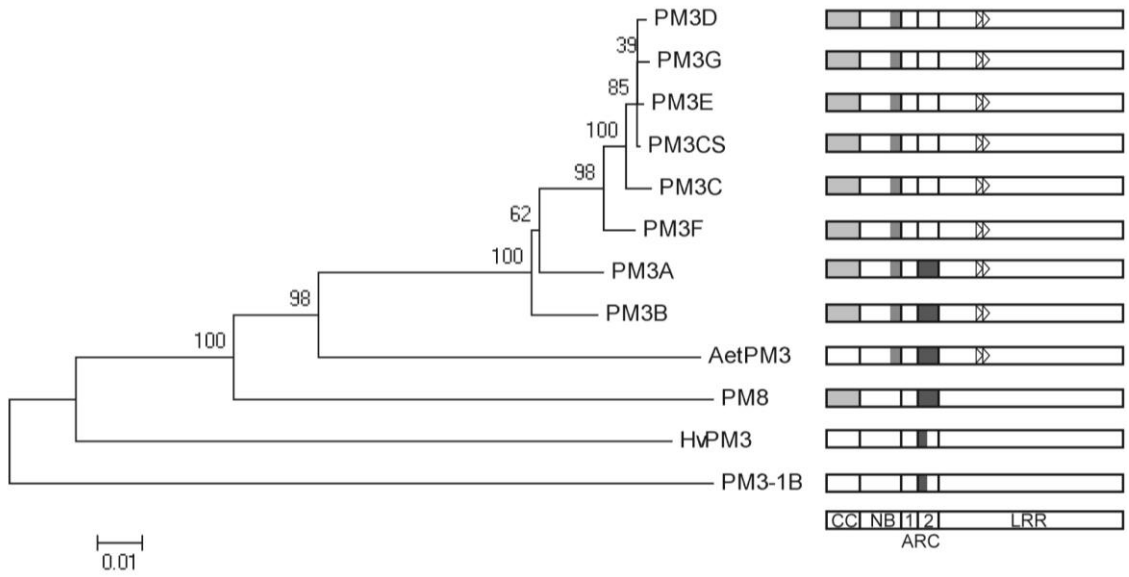


Figure 6. PM8 groups outside the wheat PM3 proteins. Neighbour-joining phylogenetic tree of full-length proteins of PM3CS, PM3A-PM3G, PM8, PM3-1B, *Aet*PM3 and *Hv*PM3.

The complete deletion option was chosen to eliminate gaps. The percentages of 1000 bootstrap replicates are depicted and the bar indicates 1% dissimilarity. On the right, the domain structure of the proteins is given and highly conserved or identical sequence blocks are shaded. The two arrows indicate the presence of the LRR6/LRR7 duplication.

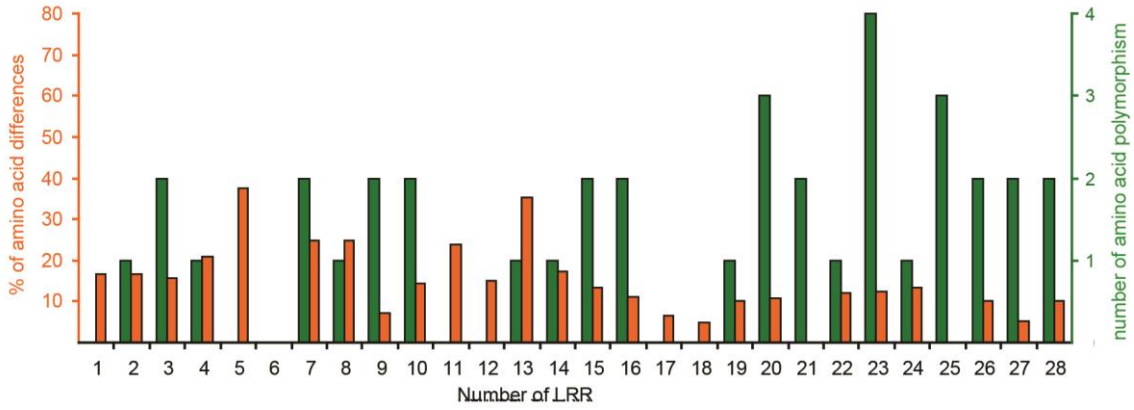


Figure 7. Amino acid changes occur prevalently in solvent-exposed residues in the leucine-rich repeats (LRRs) 20-28.

Green columns represent the number of amino acid polymorphisms among the five solvent-exposed residues of the LxxLxLxx motif for each LRR. The percentage of amino acid differences between PM8 and PM3B in the remaining positions is indicated in orange for each LRR.

Amino acid changes in the LRR domain encoded by the 17 functional *Pm3* alleles are mainly found in the predicted solvent-exposed residues close to the C-terminal end of the LRR domain and to a lesser extent at the N-terminal end and in polymorphic blocks in the middle of the domain (Bhullar *et al.* 2010, Brunner *et al.* 2010, Yahiaoui *et al.* 2006). Furthermore, Brunner *et al*. (2010) showed that polymorphic amino acids at both ends of the LRR domain are necessary for pathogen recognition specificity. Comparison of PM8 with PM3B revealed that the number of non-synonymous changes (46) in the solvent-exposed residues clearly exceeds the number of synonymous changes (16) (Table 1) and that there is an accumulation of polymorphisms found in the solvent-exposed residues of LRRs 20-28, while in LRRs 1-19 polymorphisms in solvent-exposed and non-solvent-exposed residues are present in a similar ratio (Figures 7 and S4). Thus, amino acid changes in solvent-exposed residues are concentrated in the most C-terminal region of the LRR domain if the functional PM3 proteins are compared with each other as well as in a comparison of PM3 and PM8 proteins. This strongly suggests that evolutionary forces acted in both proteins on this particular part of the LRR domain.

3.3.7 *Pm8* and *Pm3*-like genes from different grass species are a complex mosaic of common ancient haplotypes

Among the total of 54 *Pm3* alleles (functional or non-functional in resistance) known so far in wheat, *Pm3a* and P*m3b* share a unique ARC2 subdomain which is very different from the ARC2 consensus sequence (Bhullar *et al.* 2010, Yahiaoui *et al.* 2006). Interestingly, *Pm8* has exactly the same ARC2 sequence (Figures S2 and S5a). We searched for this sequence block in *Pm3*-like genes from barley, *Brachypodium*, rice, wheat and *Ae*. *tauschii* (Jia *et al.* 2013, Mayer *et al.* 2012, Vogel *et al.* 2010, Wicker *et al.* 2007a). We found one PM3-like protein from *Ae. tauschii* (*Aet*PM3) carrying this sequence block in the ARC2 subdomain (Figure S5a). Furthermore, one PM3-like

protein from *H. vulgare* (*Hv*PM3) and one protein cloned from the 1BS chromosome of hexaploid wheat (PM3-1B) had a very similar haplotype in the first half of the ARC2 subdomain (Figure S5a). The haplotype could not be found in any of the five full-length *Pm3* resistance gene analogues (RGAs) found in *T. monococcum*, *T. aestivum* and *T. turgidum*, nor in rice (Wicker *et al.* 2007a). We conclude that the specific ARC2 domain is of ancient origin and was already present in the gene pool of the Triticeae ancestor.

While the CC domains of PM8 and PM3 are highly conserved, none of the PM3-like proteins PM3-1B, *Aet*PM3 and *Hv*PM3 showed high sequence conservation in this domain compared to PM3 and PM8 (Figures 6 and S2). In contrast, the *Ae. tauschii* sequence (*Aet*PM3) shares an identical 67 amino acid long region with the PM3 alleles but not PM8 at the end of the NB domain (Figures 6, S2 and S5b). Therefore, PM8 shares with *Aet*PM3 only the unique ARC2 haplotype, but not a conserved CC nor the NB domain and the duplication of LRR6/7. In the LRR region, no conserved sequence blocks between PM8 and any of the other above analysed sequences, or any of the 54 known *Pm3* alleles, were found. These data support a model of PM8 evolution where a reshuffling of genes present at the *Pm3*/*Pm8* orthologous loci occurred before wheat and rye diverged (Figure 6). We conclude that a substantial part or most of the sequence variability in these two active resistance genes in modern wheat and rye genotypes was already present as sequence segments in the ancestors of Triticeae species and have been reused over an evolutionarily long time period.

3.4 Discussion

It is known that the activity of a number of resistance genes is actually based on two independent genes which must act together to confer resistance (Eitas and Dangl 2010). As chromosomal translocations carry hundreds of genes, it is not known if the resistances conferred by them are based on a single gene or on two or even more genes. Based on gene isolation and subsequent analysis of transient or stable transformation experiments we could show in three different genetic backgrounds that a single gene from the 1RS translocation is sufficient to confer *Pm8* race-specific resistance.

3.4.1 Unique haplotype sequence reveals orthology

Our study shows that *Pm3* and *Pm8* represent a pair of orthologous genes with a conserved resistance gene function against powdery mildew and demonstrate a high evolutionary conservation which must involve both host and pathogen components. It is generally difficult to demonstrate orthologous relationships of genes if they are members of gene families, most particularly those in gene clusters. Gene loss can result in deep paralogues which are then misinterpreted as orthologues (Wicker *et al.* 2007a). In addition, gene families in different species can evolve very differently based on unequal recombination and gene conversion, making the definition of orthologous genes impossible. These problems have mostly prevented the identification of clear resistance gene orthologues between different species in earlier studies (Li *et al.* 2012, Takahashi *et al.* 2002). Orthology between *Pm3* and *Pm8* was strongly suggested by genetic and physical mapping, but could ultimately be established by the unique haplotype conservation of the UP3 upstream sequence which is a single copy sequence in the wheat A and B genomes. It is likely that other known or yet to be

identified *Pm3* orthologues also function as powdery mildew resistance genes, but such a function remains to be demonstrated.

3.4.2 Sequence conservation suggests recognition of the same effector class by *Pm8* and *Pm3*

The highest sequence diversity in PM8 compared with PM3 was found in the solvent-exposed residues of the LRR domain at the C-terminal end. This is similar to earlier findings in other plant resistance genes: For several NB-LRR resistance genes it was shown that the solvent-exposed residues of the LRR domain are under diversifying selection (Ellis *et al.* 1999, Meyers *et al.* 1998, Parniske *et al.* 1997, Rose *et al.* 2004, Seeholzer *et al.* 2010, Yahiaoui *et al.* 2006) and the LRR domain has been shown in several cases to interact with pathogen effectors (Bergelson *et al.* 2001, Ellis *et al.* 2000b). Solvent-exposed residues were predicted to directly interact with pathogen avirulence proteins (Jones and Jones 1997, Takken and Goverse 2012). Recently, it was shown that positively selected sites are clustered at the N- and C-terminal ends of the LRR domain of the flax *L* resistance alleles and that these sites are required for interaction with AvrL567 (Ravensdale *et al.* 2012). Furthermore, race specificity was found to be determined in polymorphisms throughout the LRR domain in the flax resistance alleles *L6* and *L11* (Ellis *et al.* 2007). For PM3, we have previously shown that polymorphic amino acids reside mainly in the solvent-exposed residues of the C-terminal LRR domain and that they play a crucial role in pathogen recognition specificity (Brunner *et al.* 2010). For PM8, an accumulation of polymorphisms was found in the solvent-exposed residues mainly at C-terminal LRRs in comparison with PM3 proteins. Therefore, the LRR domain of PM8 and PM3 must have been subjected to a very similar evolutionary pressure in both rye and wheat. Given that the overall sequence is very well conserved between PM3 and PM8, it is likely that the C-terminal part of the LRR domain is very important for pathogen recognition specificity. Possibly,

the same protein is recognized by PM3 and PM8. This would also be in agreement with the data showing that there is a certain overlap in race specificity between *Pm8* and *Pm3* alleles. However, the resistance activity of *Pm8* in wheat demonstrates that an effector from wheat powdery mildew is recognized, be it directly or indirectly. Thus, although effector complements have been found to be highly specific and unique for a given pathogen species (Baxter *et al.* 2010), very similar effectors may have been conserved in mildews over more than 7 million years. This might indicate that effectors detected by PM8 and PM3 are important for mildews and are not easily lost. So far, no effector or avirulence genes have been cloned for *Bgt* or *Bgs.* It will be interesting to study and compare the molecular interactions and downstream signalling pathways of *Pm3* and *Pm8*.

3.4.3 Evolution of *Pm8* by the reshuffling of sequence blocks

There are many examples of *R* gene evolution that suggest a complex evolutionary history involving the reshuffling of sequence blocks among homologous genes (Meyers *et al.* 2005). It is known from a genome-wide study of NBS-LRR genes in *Arabidopsis thaliana* that the diversity of this gene family was generated by extensive duplication and ectopic rearrangement events (Meyers *et al.* 2003). The CC domain of wheat *Tm*MLA1 shows sequence homology to two barley *Hv*MLA subfamilies while the LRR domain is distinct from both families (Jordan *et al.* 2011). The *R* gene *N'* from *Nicotiana sylvestris* is closely related in DNA sequence to *I2* and *R3a* in the NB domain but closer to its *Capsicum* orthologue *L* in the C-terminal half of the LRR domain, suggesting a complex evolution by recombination and gene conversion (Sekine *et al.* 2012). Comparison of the *Pm3* loci from diploid, tetraploid and hexaploid wheat revealed low sequence conservation and extensive rearrangements of the *Pm3*-like genes and their up- and downstream sequences (Wicker *et al.* 2007a).

The ARC2 sequence unique for *Pm3a* and *Pm3b* among the *Pm3* alleles is present in *Pm8* and furthermore in a *Pm3*-like gene from *A. tauschii*, *AetPm3*. This sequence block shows a high number of synonymous mutations when compared with the wheat reference allele *Pm3CS* (Yahiaoui *et al.* 2006), indicating an ancient evolutionary origin. This sequence block must have evolved before wheat and rye separated. Interestingly, the PM3A/PM3B specific ARC2 domain has been shown to enhance resistance gene function and might be retained for this reason during this long time period (Brunner *et al.* 2010). The duplication of LRR6/7 which is present in the *Pm3* alleles was also found in *Aet*PM3, but is absent in *Pm8*. Considering that both *Pm8* and the *Pm3* alleles are functional *R* genes, this duplication does not seem to affect gene function. The observation of highly characteristic segments of *Pm8* and *Pm3* genes in several Triticeae species indicates that there is possibly a quite limited, natural haplotype diversity leading to functional *R* genes. It remains to be seen if approaches based on artificial resistance genes can successfully broaden useful sequence diversity for breeding (Brunner *et al.* 2010, Farnham and Baulcombe 2006).

3.4.4 Functional conservation of *Pm8* and *Pm3* gene function after host-species divergence

The ancestral gene of *Pm8* and *Pm3* must have been already present approximately 7.5 million years ago (Huang *et al.* 2002a, Huang *et al.* 2002b) before wheat and rye diverged. Given the common function of *Pm8* and *Pm3*, the ancestral gene was possibly already an active resistance gene against mildew at that time. Consequently, the resistance function has been conserved for a very long time period. In wheat, the *Pm3* gene diverged to an allelic series after its domestication (Yahiaoui *et al.* 2006). In contrast, in rye only one gene, *Pm17*, has been suggested to be a *Pm8* allele based on progeny testing of F_2 and F_3 plants (Hsam and Zeller 1997). While the race specificity of the *Pm3* alleles to wheat powdery mildew was thoroughly studied in *Pm3* differential

lines, less is known about the function of *Pm8* in rye. In agricultural ecosystems, major resistance genes introduced into an elite cultivar used by farmers are frequently only effective for a few years, as was also experienced for *Pm8* (Bennett 1984). This rapid breakdown of the *Pm8* resistance gene in the agricultural environment most likely reflects directional selection of virulence in the pathogen population due to a high selection pressure generated by the high abundance of the *R* gene. In contrast, the long life of the *Pm8* gene in natural ecosystems indicates balancing selection, where resistant and susceptible alleles are maintained over long periods of time (Brown and Tellier 2011, Stahl *et al.* 1999, Tiffin and Moeller 2006). Further studies which will identify the effector and avirulence genes from mildew are needed to clarify the molecular nature of selection.

3.5 Experimental Procedures

3.5.1 Southern blot analysis

Isolation of genomic DNA from leaf material and Southern hybridization were performed as described (Stein *et al.* 2001, Travella *et al.* 2006). For analysis with the probe UP3, genomic DNA was digested with the restriction enzyme *Hin*dIII. Probe UP3 was amplified by PCR with the primers UP3B (5'-TGGTTGCACAGACAATCC-3') and UP3A (5'-GACAAATGTGGCGTTTGC-3').

3.5.2 Amplification of *Pm8* by nested PCR and sequencing

The coding region of *Pm8* was amplified in a two-step nested PCR. A first PCR was carried out on the wheat line Kavkaz/4*Federation and rye line Petkus 91 using the primers SH32 (5'- TGCCGACCAGGCTTTGAATC-3') and N3'SP3R (5'-ACAATCAGGGATCAGGCC-3'). On the obtained PCR products, a nested PCR was performed with primer pair SH33 (5'-

TTAATTGGATCCCAATGGCAGAGCTGGTGGTC-3') and Sl_1 (5'-TATATAGTCGACGCTTCAGCTCCGGCAGGCCTG-3') adding a *Bam*HI and a *Sal*I restriction site, respectively. For all PCR reactions, the Herculase-II fusion high-fidelity DNA polymerase (600675; Agilent Technologies, http://www.chem.agilent.com/) was used.

3.5.3 Mapping of *Pm8*

For the specific detection of *Pm8*, the primer pair SH43 (5'-TGGCTTCCAACAGCCCTAGC-3') and SH46 (5'-AGGCTTTTGCACCTTCTCTC-3') was used and designated as marker *sfr43(Pm8)*. Polymerase chain reaction was performed in a total volume of 25 µl with 0.05 units/µl Taq DNA polymerase (D1806; Sigma-Aldrich, http://www.sigmaaldrich.com/) and an annealing temperature of 65°C for 30 sec.

The results of the marker *sfr43(Pm8)* on the F2 mapping population 'Federation*4/Kavkaz' x 'King II-derivative' were integrated into an existing 1RS genetic map using MAPMAKER Version 2.0 and the Kosambi mapping function for converting recombination frequencies into centimorgans.

3.5.4 Single-cell transient expression assay

The coding region of *Pm8* was amplified with the primer pair SH33/Sl_1 and ligated into the multiple cloning site of the PGY1 vector (Schweizer *et al.* 1999) under the control of the constitutively expressing 35S CaMV promoter. Seedlings of Chancellor and Federation were bombarded with 3-mg gold particles coated with 1.3 µg of the plasmid construct pGY1-*Pm8* or the empty vector control plasmid pGY1 per shot together with 1.3 µg of the reporter plasmid pUbi-GUS. The significance of the

differences between the HIs was analysed by Student's *t*-test. For a more detailed description see Methods S1.

3.5.5 Wheat transformation and analysis of transgenic *Pm8* plants

The entire 4.4-kb coding region of *Pm8* was amplified from the plasmid construct pGY1-*Pm8* using primers SH033 and TJ064 (5'-CATCATGGATCCTCACAAATCTTCTTCAGAAATCAACTTTTGTTCGCTCCGGCAGG CCTGCCTCCGC-3') thereby adding a myc-epitop tag at the 3' end of the gene and cloned into the pAHC17 vector (Christensen and Quail 1996). As a selectable marker, the phosphomannose isomerase gene was used (Reed *et al.* 2001). Transgenic plants were produced by particle bombardment of immature wheat embryos of the cultivar Bobwhite SH 98 26 essentially as described in (Brunner *et al.* 2011).

3.5.6 Quantitative real-time PCR analysis for detection of *Pm8* expression

Expression of *Pm8* was quantified in a reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) assay using a CFX96 Real-Time System C1000^{TM} Thermal cycler (Bio-Rad, http://www.bio-rad.com/). Per wheat or rye line, technical triplicates of three biological replicates each (two for rye) were analysed. Each biological replicate consisted of three pooled first leaves of 12-day-old plants. Three reference genes (*ADP*, *RLIL*, TA.6863) were included which match both the rye and wheat sequences (Table S3). Data analysis was performed using the statistical package JMP version 9.0 (SAS Institute, http://www.sas.com/). For a more detailed description see Methods S1.

3.5.7 Sequence analysis

The DNA sequences were analysed with Clone Manager Professional Suite version 8 (Sci-Ed Software, http://www.scied.com/). Sequence alignments were performed with

MEGA5 (Tamura *et al.* 2011) and corrected manually. Nucleotide diversity was calculated with DNASP version 5.0 (Librado and Rozas 2009) and phylogenetic analysis was done with MEGA5.

3.6 Acknowledgements

We thank Roi Ben-David for statistical help, A. J. Lukaszewski for providing seeds of the wheat-rye recombinant lines and Christina Cowger for supplying us with USA mildew isolates. This project was financially supported by the Swiss National Science Foundation (310030B-144081), an Advanced Investigator Grant of the European Research Council (ERC-2009-AdG 249996, Durableresistance) and the Indo-Swiss Collaboration in Biotechnology.

3.7 Supporting Information

Methods S1. Supplementary experimental procedures.

Plant material and powdery mildew infection

Wheat and rye lines which were used for Southern blot analysis, *Pm8* cloning or were tested with the *Pm8*-marker *sfr43(Pm8)* for the presence or absence of the *Pm8* gene are listed in Table S1. Wheat-rye recombinant lines T8, T9, T16, T18, 1B+14 and 1B+37 along with their parental lines Pavon 76 and Pavon 1RS.1BL (Lukaszewski 2000, Sharma *et al.* 2009) were kindly provided by Adam J. Lukaszewski (University of California, Riverside, USA). The F2 mapping population of 134 individual plants, derived from a cross between the *Pm8* carrying wheat line Federation*4/Kavkaz and a "King II" derivative wheat line, was described earlier (Mago *et al.* 2005). All plants were kept at cycles of 16 h at 20°C with light and 8 h at 16°C in the dark at a constant

relative humidity of 80%. Wheat powdery mildew isolates 07230 and 07250 originate from our own powdery mildew collection sampled in the Swiss plateau in 2007 (Brunner *et al.* 2010) and were maintained and propagated on the wheat cultivar Kanzler. Detached leaves infected with powdery mildew were kept on 0.5% phytoagar (supplemented with 30 ppm benzimidazol) plates at 17°C, 80% relative humidity and 16 h light. Powdery mildew infection and scoring were done as previously described (Kaur *et al.* 2008, Winzeler *et al.* 1991).

The isolate collection comprised 162 isolates of wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*) collected in Switzerland (Brunner *et al.* 2010), 7 isolates from the USA (USDA, North Carolina State University, USA) and 24 isolates from France (INRA, Rennes, France). Among the Swiss isolates, 33 were sampled between 1990-1998, 114 in 2007 and 15 in 2010. Leaf segment infection tests were performed as described above on *Pm3* wheat differential lines (Brunner *et al.* 2010) and on the *Pm8* wheat lines Kavkaz or Ambassador.

Homology-based cloning of *Pm8*

The primer pair UP3B (5'-TGGTTGCACAGACAATCC-3') / consLRR3B2 (5'-GCTGCAGGCATCTAGGGAGC-3') (derivative of ConsLRR3) (Figure 1a) was used for amplifying a 6.7 kb fragment from the *Pm8* wheat lines Kavkaz and Kavkaz/4*Federation and the rye lines Petkus 91 and Petkuser Winter (Srichumpa *et al.* 2005, Yahiaoui *et al.* 2004). This fragment was 100% identical in DNA sequence in all four lines. It contained an open reading frame of 3,994 bp with 91% sequence identity on the DNA level with *Pm3b*. The 5' untranslated region was shorter (2,744 bp) than the corresponding sequence of *Pm3b* (4,359 bp), showing three long as well as some smaller deletions. However, the first 184 bp which comprise the UP3 probe used for hybridisation in the Southern blot assay showed only six base-pair differences

(Figure S2). The 3' end of the gene was amplified in a 3'RACE-PCR using the primer 3'GSP1 (5'-CCATCCTTAAAGACCTTAGAA -3') (Srichumpa *et al.* 2005, Yahiaoui *et al.* 2004) (Figure 1a) and a poly-T primer. To amplify the coding region as continuous fragment, primers were designed which flank the predicted start and stop codons of the sequence (SH33 / Sl_1). Gel bands were purified with the GenElute™ Gel Extraction Kit (NA1111; Sigma-Aldrich, Buchs, Switzerland) and fragments cloned by means of the StrataClone™ Blunt PCR Cloning Kit (240207; Agilent Technologies, Basel, Switzerland).

Single-cell transient expression assay

Seedlings of Chancellor and Federation were grown at 4°C for two days followed by 7 days at growth conditions described above. Primary leaves were cut and placed on 0.8% phytoagar/30 ppm benzimidazol plates with the adaxial side up. Particle bombardment was carried out following an adapted protocol of Douchkov *et al*. (2005) and using the Biolistic PDS-1000/He System with the Hepta Adapter (Bio-Rad, Cressier, Switzerland). The *Pm8*-candidate gene together with the ß-glucuronidase (GUS) reporter gene or the GUS gene together with the empty vector (PGY1) control was delivered into wheat epidermal cells. Plates with bombarded leaves were kept at 20°C with light for 4h prior to inoculation with either the *Pm8* avirulent powdery mildew isolate 07230 or the *Pm8* virulent isolate 07250. Infected leaves were kept at 17°C with 16 h light and 80% relative humidity, treated with destaining solution ((50% glycerol, 25% lactic acid, 25% water) diluted with 2 volumes ethanol) for at least two days and fungal structures were stained with Coomassie blue (0.6% coomassie (w/v) in methanol) for a few seconds. Per construct and line and isolate, at least 150 GUS cells (50 per replicate) which were attacked by a single powdery mildew conidiospore were evaluated for absence (resistance) or presence (susceptibility) of a haustorium under the microscope to determine the haustorium index (HI) (Schweizer *et al.* 1999).

Southern blot analysis of transgenic *Pm8* plants

Genomic DNA of transgenic T_3 *Pm8* and sister lines and Bobwhite SH 98 26 was digested with *Bam*HI and *Dra*I. While *Bam*HI cuts out the entire *Pm8* coding region, *Dra*I cuts once within the gene, allowing the determination of insert copy number. Blots were hybridised with probe Pm8-intron which was amplified by PCR from pGY1-*Pm8* with primers SuB15 (5'-CAGCACGTCCTTCTATC-3') and SH50 (5'-GCTGCACATATCACAAG-3') and binds to the intron sequence of *Pm8*.

RT-qPCR analysis for detection of *Pm8* expression

RNA was extracted using the Promega SV Total RNA Isolation System kit (Z3100; Promega, Dübendorf, Switzerland) and quality was checked as described (Brunner *et al.* 2011, Risk *et al.* 2012). First-strand cDNA was synthesized from 1 µg total RNA using 2 µM (dT)20 oligomers and 100 nM of *Pm8*-specific reverse primer (Table S3), the Reverse Transcriptase SuperScript™III (18080044; Life Technologies, Zug, Switzerland) and RNaseOUT™ Recombinant RNase Inhibitor (10777019; Life Technologies) according to the manufacturer's protocol. Reference genes were chosen based on previous studies (Gimenez *et al.* 2011, Paolacci *et al.* 2009) and data on Genevestigator (Hruz *et al.* 2008) by searching for most stably expressed genes in leaves of wheat seedlings. Primers were designed for the reference genes *RLIL* and TA.6863 using Primer-blast (Ye *et al.* 2012) to target wheat as well as rye sequences (Table S3). Primers for the reference gene *ADP* were described by Gimenez *et al.* (2011). Primer and probes for the *Pm8* gene were designed based on the positions of *Pm3*-specific RT-qPCR primers (Brunner *et al.* 2011) using Primer Express® Software v2.0 (Life Technologies, Switzerland). In a reaction volume of 16 µl, 8 µl TaqMan® Fast Universal PCR Master Mix (4352046; Life Technologie, Switzerland) for detection of *Pm8* and 8 µl SsoFast EvaGreen® Supermix (172-5201; Bio-Rad, Switzerland) for the three reference genes (*ADP*, *RLIL*, TA.6863), respectively, 4 µl of sixfold diluted cDNA

and forward and reverse primers at a final concentration according to Table S3 were used. Thermocycling conditions for *Pm8* were 95°C for 20 s, followed by 40 cyles of 95°C for 4 s then 60°C for 30 s and for the reference genes 95°C for 30 s, followed by 40 cyles of 95°C for 3 s then 60°C for 5 s. Melt curves for the reference genes contained only one peak and sequencing of the fragments proved the amplification of one specific sequence. In order to check for genomic DNA contamination, cDNA samples excluding reverse transcriptase and RNaseOUT™ were included in the RT-qPCR experiment for the reference gene *RLIL*. No amplification product was obtained. Results were analysed using the program qbasePLUS V 2.3 (Biogazelle, Zwijnaarde, Belgium). Target-specific amplification efficiencies are given in Table S3. Results were normalized to the three reference genes *ADP*, *RLIL* and TA.6863. For a description of efficiency calculation and RT-qPCR set up see Risk *et al.* (2012). All data were analysed in a linear model. To fulfil assumption of equal variances, analysis was done on log-transformed values. A Tukey's honestly significant difference (HSD) test was used for mean comparison using the statistical package JMP version 9.0 (SAS Institute, Cary, NC).

Sequences of *HvPm3*, *AetPm3* and *Pm3*-1B

The *AetPm3* and *HvPm3* genes were found in a BLASTN search against the whole-genome shotgun contigs (wgs) database (https://www.ncbi.nlm.nih.gov/). The accession number for *AetPm3* is AOCO010455788.1 and for *HvPm3* CAJX010064507.1. For both genes the intron was spliced out *in silico* according to the conserved intron location in *Pm3* and *Pm8*. *Pm3*-1B was cloned in a homology-based cloning approach from wheat chromosome 1B.

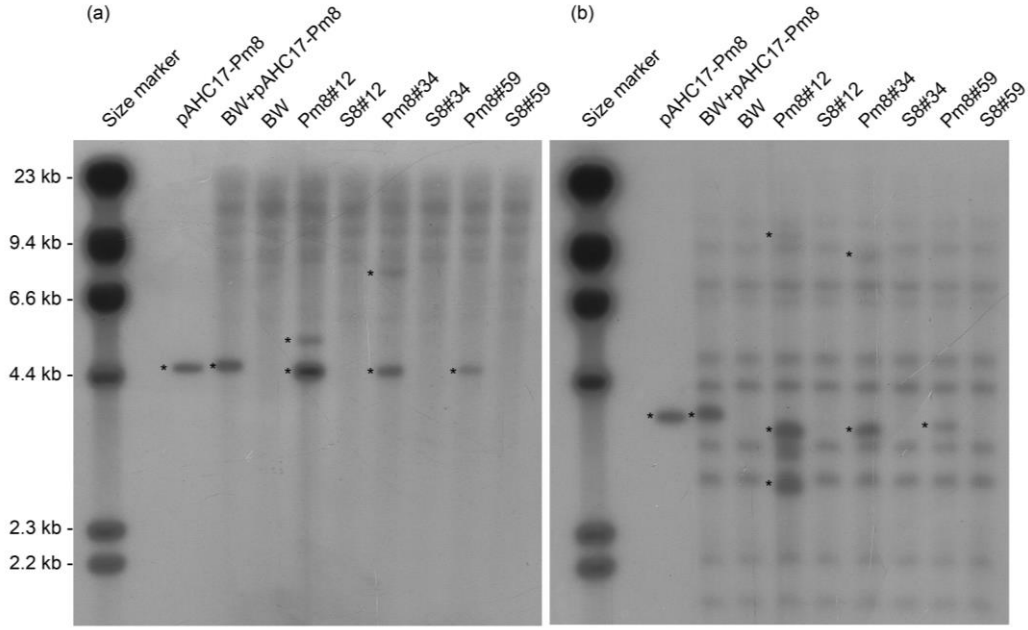


Figure S1. Southern blot analysis of T_3 transgenic *Pm8* lines. Genomic DNA was digested with restriction enzymes *Bam*HI (a) and *Dra*I (b). Blots were hybridised with a *Pm8*-derived probe (Pm8-intron) amplified from the intron sequence. Bobwhite SH 98 26 (BW) and the respective sister lines (S8: Sister line of corresponding *PM8* line selected for not carrying *Pm8*) were used as negative controls. The pAHC17 vector containing *Pm8* (pAHC17-Pm8) was used as positive control (for transformation, only the *Pm8* gene cassette had been used). *Pm8*-specific bands are indicated by asterisks. Lines Pm8#12 and Pm8#34 carry in addition to the full length transgene one disrupted *Pm8* copy (a). Line Pm8#12 carries three and Pm8#34 two copies of the *Pm8* transgene while line Pm8#59 carries a single copy (b). On the left side, the fragment lengths of the size marker are indicated.

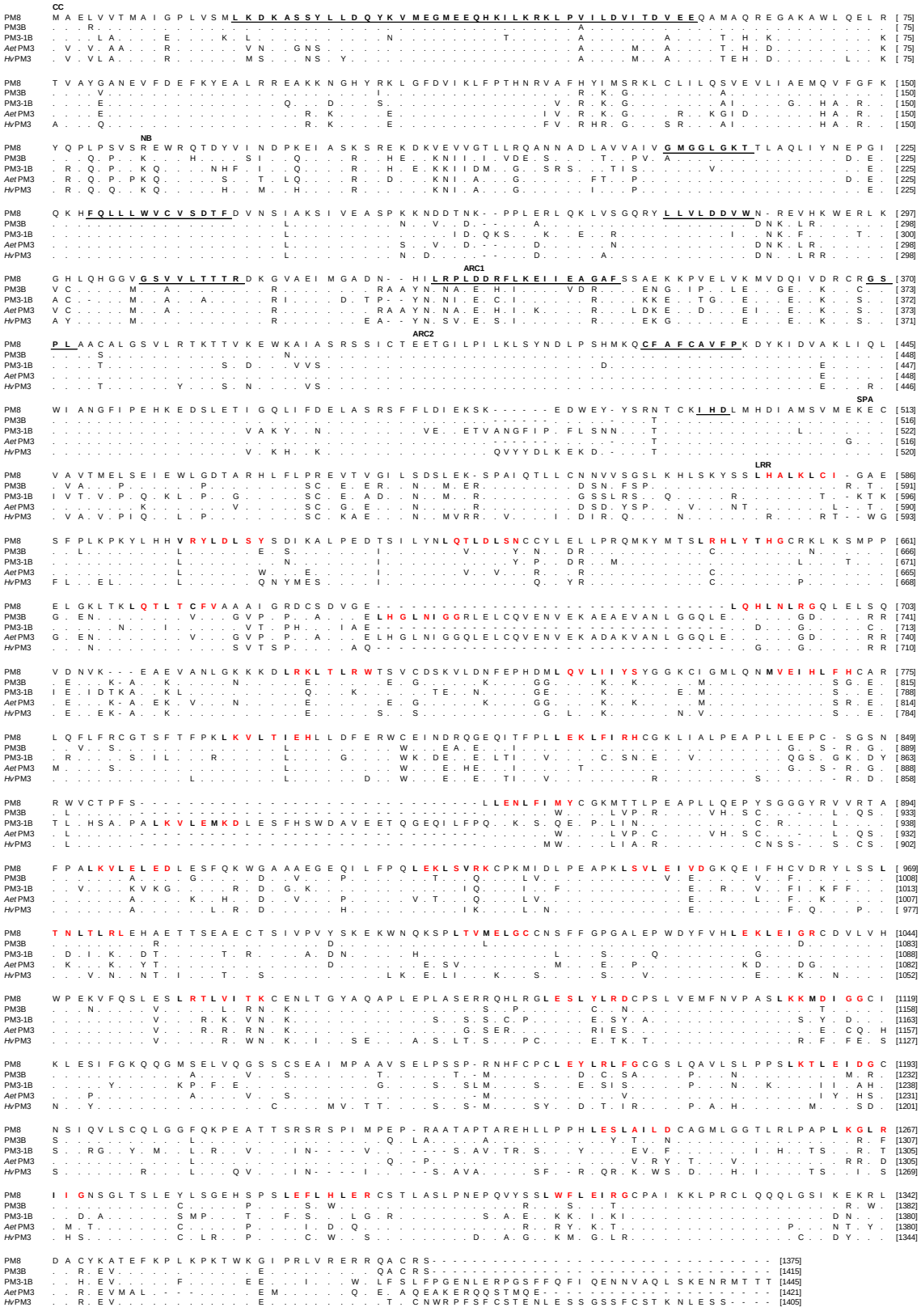


Figure S2. Amino acid sequence alignment of the proteins PM8, PM3B, PM3-1B, *Aet*PM3 and *Hv*PM3. The reference sequence PM8 is shown completely, while for PM3B, PM3-1B, *Aet*PM3

and *Hv*PM3 only polymorphic amino acids are shown (dashes represent deletions). The protein domains coiled-coil (CC; aa 1-158), nucleotide binding site (NB; aa 159-334), ARC1 (aa 335-404), ARC2 (aa 405-512) and LRR (aa 579-1414) are indicated on top of the sequence at the beginning of the domain (SPA; sequence (spacer) between ARC2 and LRR domain). The predicted coiled-coil structure in the CC domain is underlined and in bold. Conserved motifs in the NB-ARC domains are underlined and in bold in the order: Walker A/P-Loop, RNBS-A, Walker B/Kinase2, RNBS-B, RNBS-C, GLPL, RNBS-D, MHD. Aliphatic amino acids (L-positions) in the LxxLxLxx motif are in bold and the x-positions (any amino acid) are highlighted in red.

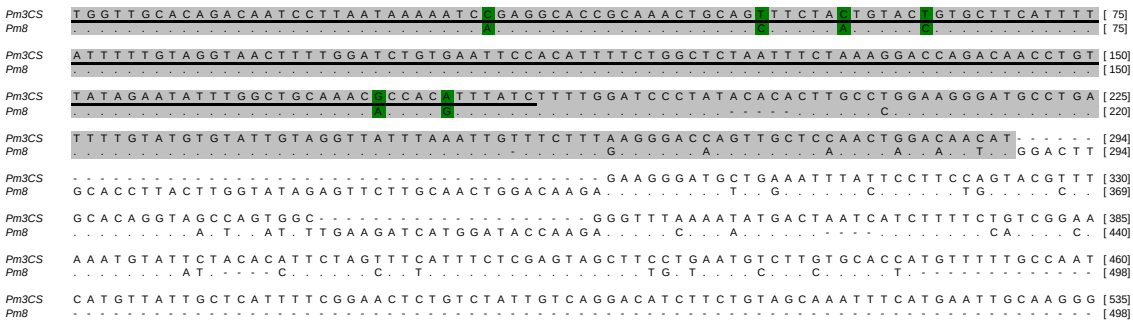


Figure S3. Comparison of the 5' region of *Pm8* with *Pm3CS* reveals high conservation of the UP3 Southern blot probe sequence. The 184 bp region (underlined) of the UP3 probe has only six nucleotide differences (highlighted in green) between *Pm3CS* and *Pm8*. High sequence conservation is found for a region of total 294 bp highlighted in grey. In contrast, the remaining 5' promoter region of shows more sequence polymorphisms (only a part of the 5' region is depicted).

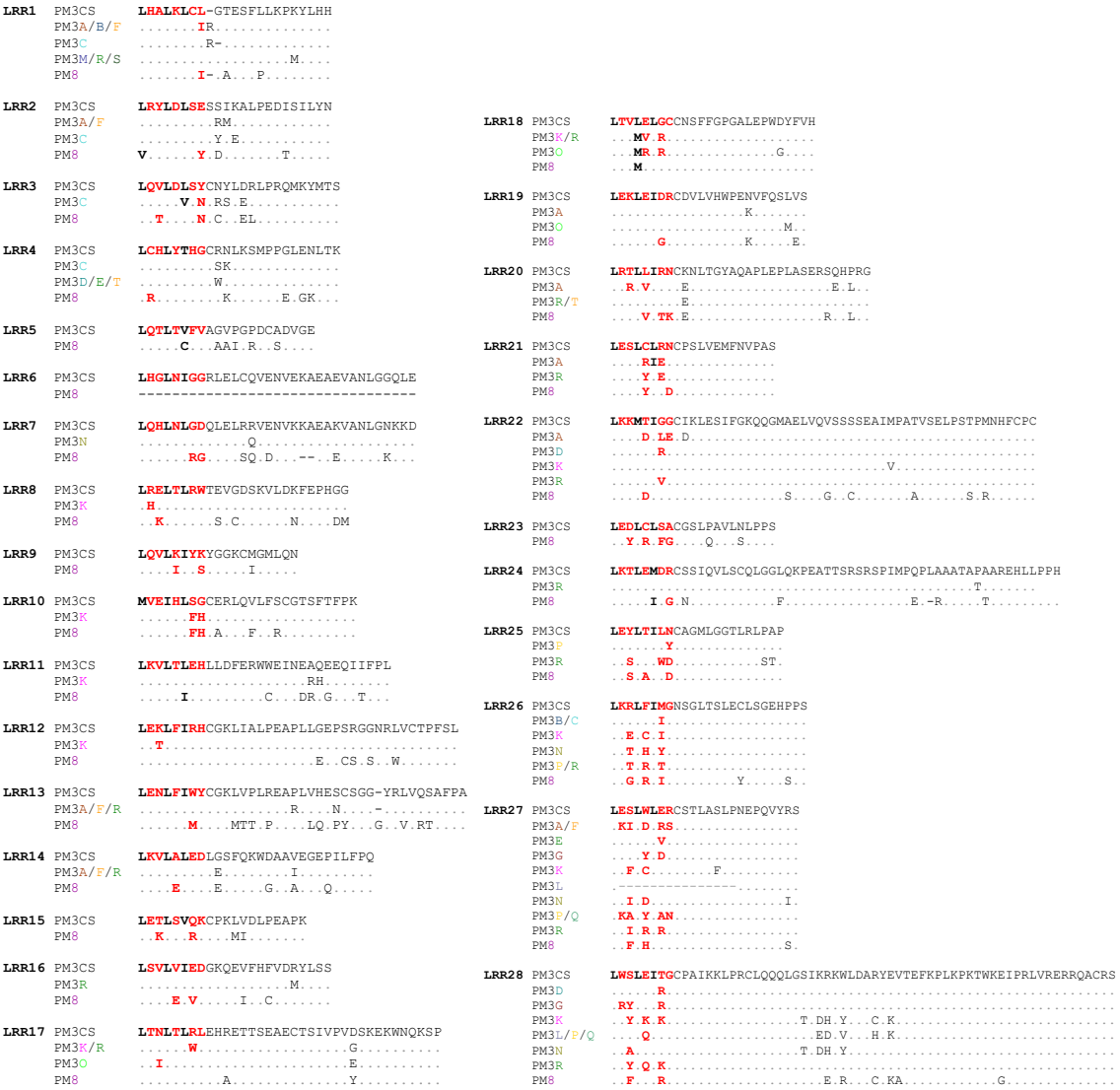
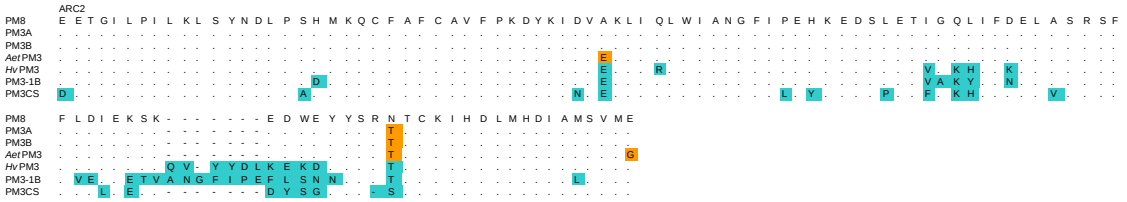


Figure S4. Amino acid polymorphisms in the LRR domains encoded by the 17 functional *Pm3* alleles (*Pm3a-g*, *k-t*) and *Pm8* are shown. Polymorphisms are indicated relative to the *Pm3CS* allele which is a non-functional *Pm3* allele and is the consensus sequence of all the *Pm3* alleles (Yahiaoui *et al.* 2006). In red, solvent exposed residues are indicated. In bold black, the conserved L positions of the LxxLxLxx motif are given. The nine LRRs 20-28 of PM8 show 20 amino acid polymorphisms in the solvent-exposed residues compared to the PM3B allele, while the other eighteen LRRs (LRR6 is missing in PM8) show only 18 differences in solvent-exposed residues. This is in strong contrast to the 27 polymorphisms in the LRRs 20-28 found outside of the solvent-exposed residues and the 71 polymorphisms in the LRRs 1-19.

(a)



(b)

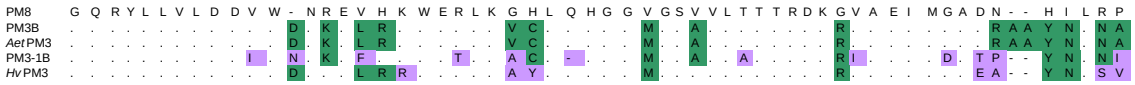


Figure S5. Highly conserved regions are found in the ARC2 and NB subdomains in PM3-like proteins from four distantly related Triticeae genomes.

a) The ARC2 subdomain is highly conserved between PM8 from rye, PM3A and PM3B from hexaploid wheat and *Aet*PM3 from the diploid *Aegilops tauschii* genome. An alignment of the full-length ARC2 subdomain sequence of PM8 (residues 402-510) with different PM3-like proteins is shown. Amino acids shared with PM8 are depicted as dots. Amino acid differences in PM3A, PM3B and *Aet*PM3 compared to PM8 are highlighted in orange. Amino acid differences in *Hv*PM3, PM3-1B (hexaploid wheat B genome) and PM3CS (hexaploid wheat A genome) compared to PM8 are highlighted in blue. Gaps are indicated as lines (-).

b) *Aet*PM3 and PM3B share a sequence block at the C-terminal end of the NB domain which is not conserved in PM8. Residues 275 to 331 (position in PM8) are shown. In green, amino acids shared among the PM3-like proteins but not with PM8 are indicated. All other amino acid differences compared to PM8 are highlighted in purple. For complete sequence alignment see Figure S2.

Table S1. Association of the *Pm8*-candidate gene marker *sfr43(Pm8)* with *Pm8* in 1BL.1RS wheat and in rye lines.

	Genotype	*Pm8* status	*sfr43(Pm8)*[f]	seed origin
wheat	Ambassador	*Pm8*	+	ACW Changins
1BL.1RS	Apollo	*Pm8*	+	ART Reckenholz
	Benno	*Pm8*	+	ART Reckenholz
	Bobwhite SH 98 56	*Pm8*	+	CIMMYT Mexico
	Clement	*Pm8*	+	ART Reckenholz
	Dakota	*Pm8*	+	ART Reckenholz
	Disponent	*Pm8*	+	ART Reckenholz
	Florida	*Pm8*	+	ART Reckenholz
	Granada	*Pm8*	+	AELF Moosburg
	Kavkaz	*Pm8*	+	ART Reckenholz
	Kavkaz/4*Federation	*Pm8*	+	ART Reckenholz
	Kronjuwel	*Pm8*	+	ART Reckenholz
	Pavon 1RS.1BL	*Pm8*	+	University of California
	Petrus	*Pm8*	+	ART Reckenholz
	Sarhad 82	*Pm8*	+	University of Sydney
	Stuart	*Pm8*	+	NRC Canada
	Tarso	*Pm8*	+	ART Reckenholz
	Veery#5	*Pm8*	+	University of Sydney
	Veery#6	*Pm8*	+	University of Sydney
	Weique	*Pm8*	+	IPK Gatersleben
	CIGM98.770-1[a]	*Pm8*	+	USDA
	CIGM98.773-1[a]	*Pm8*	+	USDA
	CIGM98.777-1[a]	*Pm8*	+	USDA
wheat	Amigo[b]	no *Pm8*	-	USDA
without	Caribo[c]	no *Pm8*	-	ART Reckenholz
1BL.1RS	Chul/8*Chancellor[d]	no *Pm8*	-	ART Reckenholz
(normal				
1B)	Chinese Spring[c]	no *Pm8*	-	ART Reckenholz
	Federation	no *Pm8*	-	University of Sydney
	Pavon	no *Pm8*	-	University of California
	Bobwhite SH 98 26	no *Pm8*	-	CIMMYT Mexico
rye	Petkus 91	unknown[e]	+	IPK Gatersleben
	Petkus II	unknown[e]	-	IPK Gatersleben
	Petkuser Winter	unknown[e]	+	IPK Gatersleben
	Imperial	no *Pm8*	-	IPK Gatersleben
	King II	no *Pm8*	-	CSIRO Australia
	Blanco	unknown	-	ACPFG Australia

[a]*T. durum*

[b]carries *Pm17*, the powdery mildew resistance gene proposed to be allelic to *Pm8* (Hsam and Zeller 1997).

[c]carries *Pm3CS*

[d]carries *Pm3b*

[e]Likely to carry *Pm8*, since 'Petkus' is the donor of the 1RS chromosome arm in 1BL.1RS translocation lines.

[f]+: 662 bp fragment amplified from *Pm8*-candidate promoter and coding region
- : no fragment amplified

Table S2. Haustorium indices (%) with *Bgt* 07230 and 07250 reported in Figure 4a.

		HI (%) 07230				HI (%) 07250			
construct	line	rep1	rep 2	rep 3	mean	rep1	rep 2	rep 3	mean
empty vector	Federation	68	61	68	66	61	56	64	60
empty vector	Chancellor	67	68	67	67	69	67	62	66
Pm8 candidate	Federation	26	23	33	27	64	56	66	62
Pm8 candidate	Chancellor	18	29	30	26	66	69	65	67

Table S3. Primers and probe used for the RT-qPCR assay.

Target gene (UniGene)	Gene name	5'-3' Sequence; modifications	Primer/Probe concentration nM	PCR efficiency (E) r^2 of calibration curve Slope	Amplicon length bp	Reference
	Pm8	F CTGGGCAGCATCAAGGA R CCGCTCACGGACTAGCCTC Probe VIC-CCTGCTATAAAGCAACG-MGBNFQ	250 250 250	E= 98% r^2 = *0.999* Slope = -3.375	108	(Brunner *et al.* 2011); this work
TA.2291	*ADP*	F CTGGAGCACGAAGCTGCAG R CGAGTGCTGGAGCTTGCAGT	400 400	E= 105% r^2 = *0.998* Slope = -3.218	80	(Gimenez *et al.* 2011)
TA.2776	*RLIL*	F CTTAAAGTCTTAGCTGGCAA R CTTGATGATTGCCTTCAGGT	400 400	E= 97% r^2 = *0.989* Slope = -3.400	150	(Paolacci *et al.* 2009); this work
TA.6863	TA.6863	F GCAGGGTCAGGAAGATATTGG R GAATCTGGCCTACGGTTGAT	400 400	E= 106% r^2 = *0.997* Slope = -3.191	105	this work

4. Powdery mildew resistance gene *Pm8* derived from rye is suppressed by its wheat ortholog *Pm3*

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4.1 Summary

The powdery mildew resistance gene *Pm8* derived from rye is located on a 1BL.1RS chromosome translocation in wheat. However, some wheat lines with this translocation do not show resistance to isolates of the wheat powdery mildew pathogen avirulent to *Pm8* due to an unknown genetically dominant suppression mechanism. Here we show that lines with suppressed *Pm8* activity contain an intact and expressed *Pm8* gene. Therefore, the absence of *Pm8* function in certain 1BL.1RS containing wheat lines is not the result of gene loss or mutation but is based on suppression. The wheat gene *Pm3*, an ortholog of rye *Pm8*, suppressed *Pm8*-mediated mildew resistance in lines containing *Pm8* in a transient single-cell expression assay. This result was further confirmed in transgenic lines with combined *Pm8* and *Pm3* transgenes. Expression analysis revealed that suppression is not the result of gene silencing, either in wheat 1BL.1RS translocation lines carrying *Pm8* or in transgenic genotypes with both *Pm8* and *Pm3* alleles. In addition, a similar abundance of the PM8 and PM3 proteins in single or double homozygous transgenic lines suggested that a post-translational mechanism is involved in *Pm8* suppression. Co-expression of *Pm8* and *Pm3* genes in *N. benthamiana* leaves followed by co-immunoprecipitation analysis showed that the two proteins interact. Therefore, the formation of a heteromeric protein complex might result in inefficient or absent signal transmission for defence reaction. These data provide a molecular explanation for resistance gene suppression in certain genetic backgrounds and suggest ways to circumvent it in future plant breeding.

4.2 Introduction

The expanding demand for agricultural products requires a substantial increase in productivity of crop plants over future decades. There is a great potential to improve productivity by reduction of yield losses caused by pathogens. The deployment of resistance (*R*) genes is an effective, environmentally sustainable and economical approach to protect plants from pathogen attacks. *R* gene products directly or indirectly recognize molecules of the pathogen and this recognition triggers a defence reaction often accompanied by a hypersensitive response leading to cell death and inhibition of further pathogen spread (Dodds and Rathjen 2010).

Major resistance genes in agriculture break down rapidly due to strong selection for virulent pathogen mutants. Wild crop relatives provide a rich resource for the identification of new, effective disease resistance genes that can be used in plant breeding. Specifically, wild grasses as well as wild wheat species with lower ploidy levels are frequently used in wheat breeding (Baum *et al.* 1992). These genetic resources represent a large gene pool compared to elite wheat cultivars with an overall low genetic diversity. Several powdery mildew (*Pm*) resistance genes have been identified in wheat relatives and introgressed into hexaploid wheat (Hsam and Zeller 2002). In this respect cereal rye is a particularly valuable genetic resource, providing not only rust and powdery mildew resistance but also agronomic traits such as yield increase and wide adaptation ability. The best known and widely deployed powdery mildew resistance gene from rye, located on a 1BL.1RS translocation of hexaploid wheat, is *Pm8*. It was derived from the introgression of the 1RS rye chromosome from rye cultivar ‘Petkus’ into hexaploid wheat in the 1930s (Zeller 1973). Besides *Pm8*, there are a number of rust resistance genes present in the 1RS translocation.

However, not all wheat lines carrying this translocation and expressing its rust resistance, therefore assumed to also carry *Pm8*, show resistance to *Pm8* avirulent powdery mildew isolates due to the presence of a suppressor in wheat. Hanusova *et al*. (1996) described a single dominant gene on chromosome 7D that was responsible for *Pm8* suppression whereas Ren *et al*. (1996) observed a genetic association of *Pm8* suppression with the presence of a gliadin protein band encoded by a gene on chromosome 1AS. The gliadin storage gene is located in close proximity to the *Pm3* locus and McIntosh *et al*. (2011) showed that *Pm8* suppression is genetically correlated with a *Pm3*-specific marker in a 5' sequence of *Pm3* located 4.4 kb upstream of the start codon. We recently cloned *Pm8* and found that it is a rye ortholog of the *Pm3* wheat powdery mildew resistance gene located on wheat chromosome 1AS (Hurni *et al.* 2013). Both genes code for coiled-coil (CC), nucleotide-binding site, ARC1 and ARC2 (NB-ARC) and leucine-rich-repeat (LRR) domain proteins and mediate race-specific resistance against powdery mildew in wheat (Hurni *et al.* 2013, Yahiaoui *et al.* 2004).

Resistance gene suppression is neither limited to *Pm8* nor to powdery mildew resistance, but is a common phenomenon in plant breeding. In wheat breeding, it is often observed when chromosomes from wild wheat or relatives with lower ploidy level are introgressed into hexaploid wheat (Hsam and Zeller 2002, McIntosh *et al.* 2011). Numerous genetic studies described the partial or complete suppression of rust resistance genes in synthetic wheat (Boyd 2005). The studied suppressor genes were found to be resistance gene specific and their genetic locations differed (Assefa and Fehrmann 2004, Kema *et al.* 1995, Ma *et al.* 1995). Besides the suppression of powdery mildew and rust resistance genes in wheat, suppression to tan spot was observed (Siedler *et al.* 1994). Furthermore, resistance gene suppression was also described in other crop species; in oat against crown rust resistance (Wilson and

McMullen 1997), in soybean against soybean rust resistance (Garcia *et al.* 2011) and in potato against late blight resistance (Ordoñez *et al.* 1997). Thus, the numerous reports of suppression of resistance to diverse pathogens in several crop species indicate that resistance gene suppression is a common phenomenon that seriously affects the efficiency of resistance breeding.

In this study, we identified the dominant suppressor of *Pm8* as the previously cloned *Pm3* gene. We show that the *Pm3* gene is sufficient to suppress *Pm8* race-specific resistance and that both the suppressing gene *Pm3* and the suppressed gene *Pm8* are expressed. Transgenic lines homozygous for both genes had PM3 and PM8 protein levels that were very similar to the homozygous sister lines with the respective single genes. Therefore, *Pm3* is the suppressor of *Pm8* and suppression most likely involves post-translational processes such as the formation of non-functional protein complexes.

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4.3 Results

4.3.1 An intact *Pm8* gene is present in lines with suppressed *Pm8*-mediated resistance

The suppression of *Pm8*-mediated resistance in certain wheat 1BL.1RS translocation lines correlated with the presence of the *Pm3* locus on wheat chromosome 1AS (McIntosh *et al.* 2011). Based on the recent isolation of the *Pm8* gene (Hurni *et al.* 2013) we wanted to study the suppression at the molecular level in six wheat lines all containing the rye translocation. The four wheat lines Kavkaz/4*Federation, Benno, Ambassador and Veery#6 showed no suppression of *Pm8* as indicated by their resistance against wheat powdery mildew (*Blumeria graminis* f.sp. *tritici* (*Bgt*)) isolate 07230 which is avirulent to *Pm8* (Table S1). In contrast, the two lines Veery#5 (McIntosh *et al.* 2011) and Florida (Hanusova *et al.* 1996) were susceptible, indicating *Pm8* gene suppression (Figure S1a, Table 1). All lines were tested for the presence of the *Pm8* gene by PCR amplification and sequencing. Indeed, in all six lines the complete and identical *Pm8*-coding sequence was present (Table 1). To check if the wheat *Pm3* gene is present in these lines, we used a nested PCR strategy which has been used earlier to clone all 54 *Pm3* alleles known so far (Bhullar *et al.* 2010, Srichumpa *et al.* 2005). PCR amplification revealed that the lines Florida and Veery#5 contained the coding sequences of the *Pm3* alleles *Pm3CS* (Yahiaoui *et al.* 2006) and *Pm3_8152* (Bhullar *et al.* 2010), respectively. No *Pm3* alleles could be amplified from the four wheat lines not showing suppression of *Pm8* (Table 1). In a next step, we checked if the *Pm8*-mediated resistance in line Kavkaz/4*Federation can be suppressed by crossing it with the hypothetical suppressor line Chinese Spring (*Pm3CS*). F_4 segregants homozygous for both *Pm3CS* and *Pm8* (24 F4 individuals were tested) were susceptible to *Bgt* isolate 07230 whereas the segregants homozygous for *Pm8* only (24 F4 individuals were tested) were resistant (Figure S1b).

Therefore, suppression of *Pm8* correlated with the presence of a *Pm3* allele and the lack of *Pm8*-mediated powdery mildew resistance in certain wheat lines is indeed not caused by mutant versions or complete absence of *Pm8*.

Table 1. The *Pm8* gene and a *Pm3* allele are present in lines suppressing *Pm8*-mediated resistance.

Wheat line	*Pm8*-mediated resistance [a]	*Pm8*[a]	*Pm3*[a]	Source of germplasm
Kavkaz/4*Federation	+	+	-	Agroscope in Reckenholz
Benno	+	+	-	Agroscope in Reckenholz
Ambassador	+	+	-	Agroscope in Changins
Veery#6	+	+	-	University of Sydney
Veery#5	-	+	*Pm3_8152*	University of Sydney
Florida	-	+	*Pm3CS*	Agroscope in Reckenholz

[a]+/-: presence/absence of the phenotype or gene

4.3.2 *Pm8* resistance is suppressed by *Pm3CS* in a transient expression assay in wheat leaf epidermal cells

So far, a correlation between the presence of the *Pm3* alleles *Pm3CS* and *Pm3_8152* and suppression of *Pm8*-mediated resistance was observed. To test whether the *Pm3* gene is required and also sufficient for *Pm8* suppression, we carried out a single-cell transient expression assay. The susceptible *Pm3* allele *Pm3CS* was transiently expressed in leaf epidermal cells of wheat lines with *Pm8*-based resistance. As a control we used cultivar Federation which is highly susceptible to powdery mildew. After bombardment, leaf segments were inoculated with *Bgt* isolate 07230 which is avirulent to *Pm8* (Table S1). To measure the level of powdery mildew susceptibility, the haustorium index (HI) was determined by counting the percentage of transformed

epidermal cells penetrated by a single powdery mildew spore to form a haustorium. Transient expression of the *Pm3CS* allele lead to a significant increase in the HI in all three tested lines Kavkaz/4*Federation (Kav/4*Fed), Benno and Ambassador compared to the empty vector control (Figure 1, Table S2). This result demonstrated that *Pm3CS* suppressed *Pm8*-mediated resistance and, therefore, is sufficient to suppress *Pm8*-mediated powdery mildew resistance. Furthermore, it confirms that *Pm3* is the dominant suppressor gene of *Pm8*, *SuPm8*.

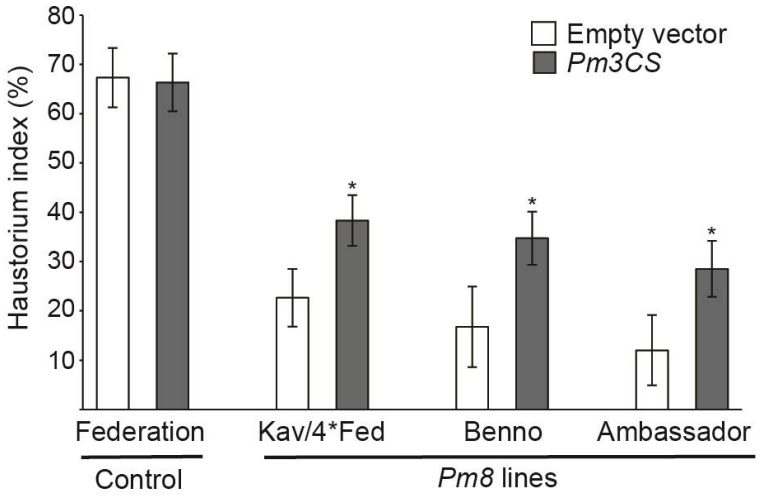

Figure 1. *Pm8*-mediated resistance in three different wheat-rye translocation lines is suppressed by the *Pm3CS* gene.

The haustorium index, a measure of susceptibility, was significantly higher when leaf segments of wheat-rye translocation lines Kavkaz/4*Federation (Kav/4*Fed), Benno and Ambassador were bombarded with the susceptible *Pm3* allele *Pm3CS* compared to the empty vector control (Student's *t*-test, $*P < 0.05$). Leaf segments were infected with the *Pm8* avirulent *Bgt* isolate 07230. No significant difference was found when *Pm3CS* was transiently expressed in the wheat line Federation which does not contain *Pm8,* compared to the empty vector control (Student's *t*-test, $P = 0.85$). The haustorium indices represent the mean of three independent biological replicates (four for Benno and Ambassador) and error bars indicate standard deviations.

4.3.3 *Pm8* resistance is suppressed by three functional *Pm3* alleles in stable transgenic wheat lines

The single-cell transient expression assay demonstrated that *Pm8*-mediated resistance is suppressed by the susceptible *Pm3* allele *Pm3CS* which is also present in the *Pm8*-suppressed cultivar Florida (Table 1). In the *Pm8*-suppressed cultivar Veery#5 a different susceptible *Pm3* allele, *Pm3_8152*, is present (Table 1). Furthermore, McIntosh *et al*. (2011) and Hao *et al*. (2012) showed that *Pm8* is suppressed in a cross carrying the *Pm3* allele *Pm3a* based on molecular marker data. This suggests that *Pm3* alleles other than *Pm3CS* are able to suppress *Pm8*-mediated resistance as single genes. To analyse this further as well as to demonstrate specific suppression of *Pm8* in a stable transgenic system, we crossed previously developed transgenic *Pm3* lines (Brunner *et al.* 2012) (Stirnweis *et al*., 2014a, accompanying manuscript) with a transgenic line carrying *Pm8* (Hurni *et al.* 2013). Such crosses permit the study of *Pm8* suppression in the presence or absence of a *Pm3* allele in the same genetic background. Both the *Pm3* and *Pm8* transgenes are in the genetic background of a powdery mildew susceptible selection of wheat cultivar Bobwhite (Bobwhite SH 98 26). Furthermore, with a single hemagglutinin (HA) epitope tag fused C-terminally to the transformed *Pm3* gene constructs and a c-myc (myc) epitope tag in the *Pm8* construct, protein analyse can be performed.

The *Pm8* transgenic line Pm8#59 (Hurni *et al.* 2013) was crossed with the transgenic lines Pm3a#1, Pm3f#1 (Brunner *et al.* 2012) and $Pm3b_{HA}$ (Stirnweis *et al*., 2014a, accompanying manuscript). Expression of all transgenes is driven by the strong and constitutively active maize ubiquitin promoter. All lines showed expression of the transgene and mediated race-specific powdery mildew resistance in infection tests (Brunner *et al.* 2012, Hurni *et al.* 2013) (Stirnweis *et al*., 2014a, accompanying manuscript). For all three gene combinations one cross was made. In the F_3

generation, segregant lines homozygous for the respective *Pm3* allele as well as *Pm8* were selected. In addition to these double-homozygous lines (*Pm8*/*Pm3a, b or f*) homozygous sister lines carrying only *Pm8* (*Pm8*/(Δ*Pm3a*, *b* or *f*)) or the respective *Pm3* allele ((Δ*Pm8*)/*Pm3a*, *b* or *f*) were selected and propagated to the F_4 generation for analysis.

To check if *Pm8*-mediated resistance was suppressed in the double-homozygous lines, each line was tested with three different *Pm8*-avirulent *Bgt* isolates along with their *Pm8* homozygous sister lines. In total, we used five *Bgt* isolates which were avirulent on the parental line Pm8#59 with the *Pm8* transgene but virulent on one or more of the parental *Pm3* trangenic lines, allowing the functionality of *Pm8* to be determined (Table S1). The *Pm8* homozygous sister lines showed the expected complete resistance to the avirulent *Pm8 Bgt* isolates (*AvrPm8*) (Figure 2 and S2). Interestingly, the double-homozygous lines *Pm8*/*Pm3a* and *Pm8*/*Pm3f* showed intermediate susceptibility to *Bgt* isolates AK3-11 and C3-1 (29-53% infected leaf area) but remained completely resistant to *Bgt* isolate 10001 (Figure S2a and S2c). The double-homozygous line *Pm8*/*Pm3b* was susceptible to *Bgt* isolate AK3-11 (89% infected leaf area), slightly susceptible to *Bgt* isolate 97011 (18% infected leaf area) but completely resistant when infected with *Bgt* isolate 98229 (Figure 2 and S2b). Thus, *Pm3a*, *Pm3f* and *Pm3b* suppressed the *Pm8*-mediated resistance either completely, partially or not at all in an isolate-specific way (Figure 3). Therefore, even though *Pm8* is more strongly expressed in the Pm8#59 transgenic line than in wheat lines with the rye translocation (Hurni *et al.* 2013), *Pm3* transgenes are able to suppress *Pm8*-mediated resistance to certain *Pm8*-avirulent *Bgt* isolates.

In a next step, we wanted to study the reciprocal situation, *i.e.* if *Pm3*-mediated resistance is suppressed by *Pm8.* For this reason, the *Bgt* isolates 07250 and 10004 which are virulent on the Pm8#59 transgenic line but avirulent on the *Pm3* lines

Pm3a#1, Pm3f#1 and $Pm3b_{HA}$ were used (Table S1). The homozygous *Pm3* sister lines as well as the double-homozygous lines were completely resistant (Figure 2 and S2). However, line *Pm8*/*Pm3f* was slightly less resistant than its sister line (Δ*Pm8*)/*Pm3f* (11% infected leaf area versus 1%; Figure S2c), indicating a weak interference of *Pm8* with the *Pm3f* resistance response. This suggests that the resistance suppression is not necessarily reciprocal.

We conclude that all three tested *Pm3* alleles (*Pm3a*, *Pm3b* and *Pm3f*) were able to suppress *Pm8*-mediated resistance in transgenic lines and it is likely that all *Pm3* alleles are able to suppress *Pm8*-mediated resistance after infection with certain powdery mildew isolates. Interestingly, suppression varied quantitatively between the different *Bgt* isolates tested and was therefore race-specific (Figure 3 and S2). Thus, resistance suppression of *Pm8* is a quantitative effect that depends on the combination of isolate and the *Pm3* allele that is combined with *Pm8*.

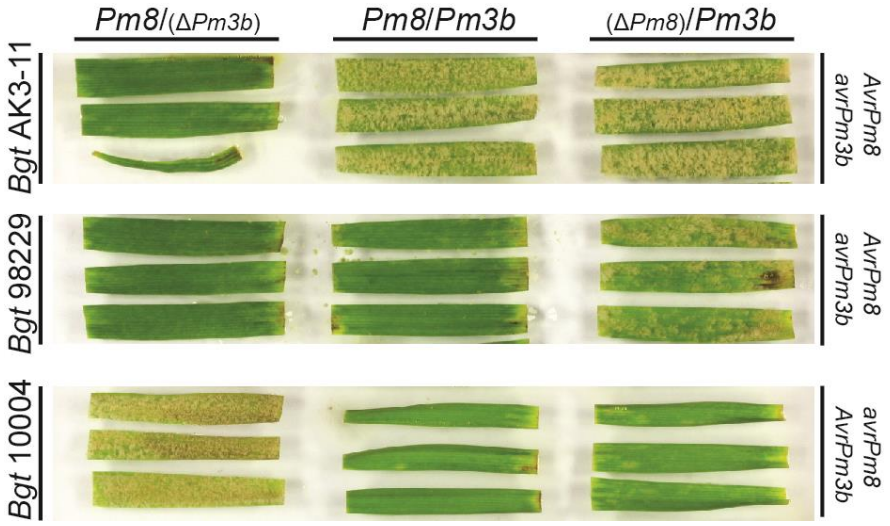

Figure 2. *Pm3b* suppresses *Pm8*-mediated resistance in lines with the two combined transgenes.

F_4 plants of the double-homozygous line *Pm8*/*Pm3b* were tested with isolates differing in resistance response on their parents with single transgenes. *Bgt* isolates AK3-11 and 98229 were avirulent on the *Pm8* homozygous sister line *Pm8*/(Δ*Pm3b*) but virulent on the *Pm3* homozygous sister line (Δ*Pm8*)/*Pm3b*. The double-homozygous *Pm8*/*Pm3b* line showed complete suppression of *Pm8*-mediated resistance when infected with *Bgt* isolate AK3-11. No *Pm8* suppression was observed with isolate 98229. Furthermore, no suppression of the *Pm3b*-mediated resistance by *Pm8* was observed when infected with the *Pm3b* avirulent isolate 10004. Images were taken eight days after infection and depict representative results from the infection tests reported in Figure S2. The presence of the avirulence (*Avr*) and virulence (*avr*) genes relevant for this study is indicated on the right side of the infection pictures.

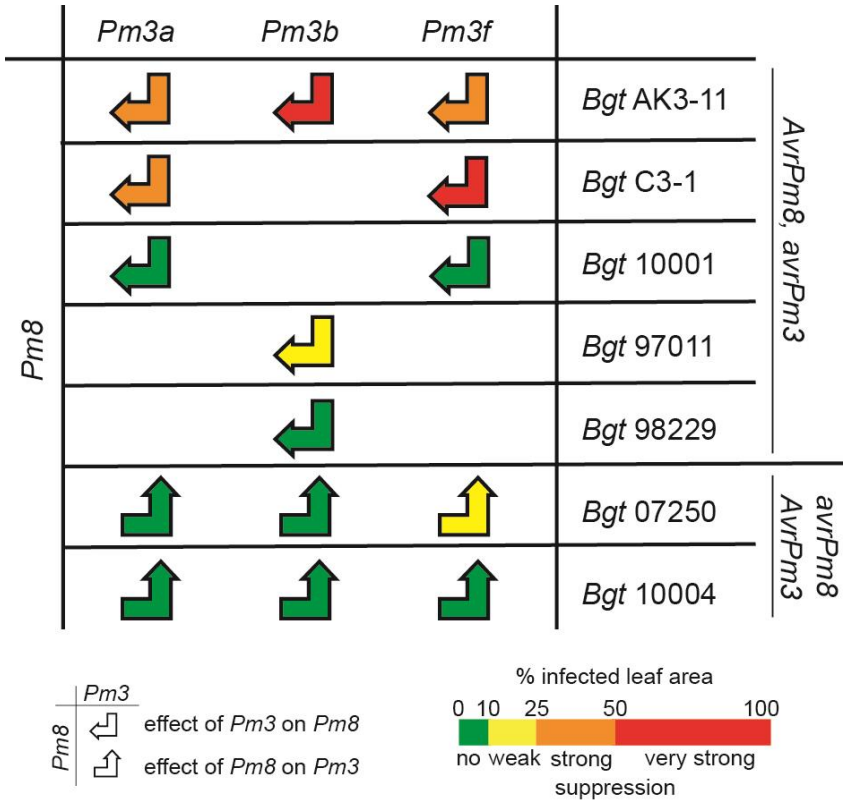

Figure 3. Suppression of *Pm8*-mediated resistance by *Pm3* in transgenic lines is quantitative and *Bgt* isolate dependent.

The data were obtained by infection tests made with five *Pm8*-avirulent, *Pm3*-virulent *Bgt* isolates AK3-11, C3-1, 10001, 97011 and 98229 and two *Pm3*-avirulent, *Pm8*-virulent isolates 07250 and 10004 on the double-homozygous transgenic lines (Figure S2). The arrowheads indicate the direction of suppression and the colour indicates suppression intensity. Arrowheads pointing to the left indicate the effect of the respective *Pm3* allele on the *Pm8*-mediated resistance, whereas arrowheads pointing upwards indicate the effect of *Pm8* on *Pm3*-mediated resistance. Intermediate to strong suppression of the *Pm8*-mediated resistance caused by the *Pm3a*, *Pm3b* and *Pm3f* alleles was found with isolates AK3-11 and C3-1, but not with isolates 10001 or 98229, and there was weak suppression with isolate 97011. Presence (*Avr*) or absence (*avr*) of relevant avirulence genes is indicated beside the powdery mildew isolate names.

4.3.4 *Pm8* suppression is neither due to changes in *Pm8* gene expression nor protein abundance

Suppression of *Pm8* could be due to transcriptional or post transcriptional silencing. Therefore, in a first step we measured the *Pm8* expression level in a reverse transcription, quantitative real-time PCR (RT-qPCR) assay in non-transgenic lines showing suppression. The expression level was determined in both uninfected and infected plants of the *Pm8*-suppressed lines Veery#5 and Florida and the *Pm8*-non-suppressed lines Benno and Veery#6 (8 hours and 24 hours post infection with the *Pm8*-avirulent *Bgt* isolate 07230). Independent of treatment, timepoint or genotype *Pm8* was expressed to an equivalent level in all four wheat 1BL.1RS lines, irrespective of the *Pm8* resistance phenotype (Figure 4a). Furthermore, we analysed the expression levels of *Pm8* and *Pm3CS* in the homozygous and double-homozygous progeny of the cross Chinese Spring with Kavkaz/4*Federation without infection and 24 hours post infection. *Pm8* as well as *Pm3CS* were expressed in the double-homozygous line *Pm3CS*/*Pm8* to an equivalent level as in the homozygous lines (Δ*Pm3CS*)/*Pm8* and *Pm3CS*/(Δ*Pm8*) (Figure S3). We also analysed the expression level of *Pm8* under the strong ubiquitin promoter in the double-homozygous transgenic lines *Pm8*/*Pm3a*, *Pm8*/*Pm3b* and *Pm8*/*Pm3f* and the respective *Pm8* homozygous sister lines. Consistently, no significant difference in *Pm8* expression level between the double-homozygous and the corresponding homozygous sister lines was found (Figure 4b). Furthermore, there was also no significant difference in *Pm3* expression level under the strong ubiquitin promoter between the double-homozygous lines and corresponding *Pm3* homozygous sister lines (Figure 4b). These results suggest that suppression does not occur at the transcriptional level.

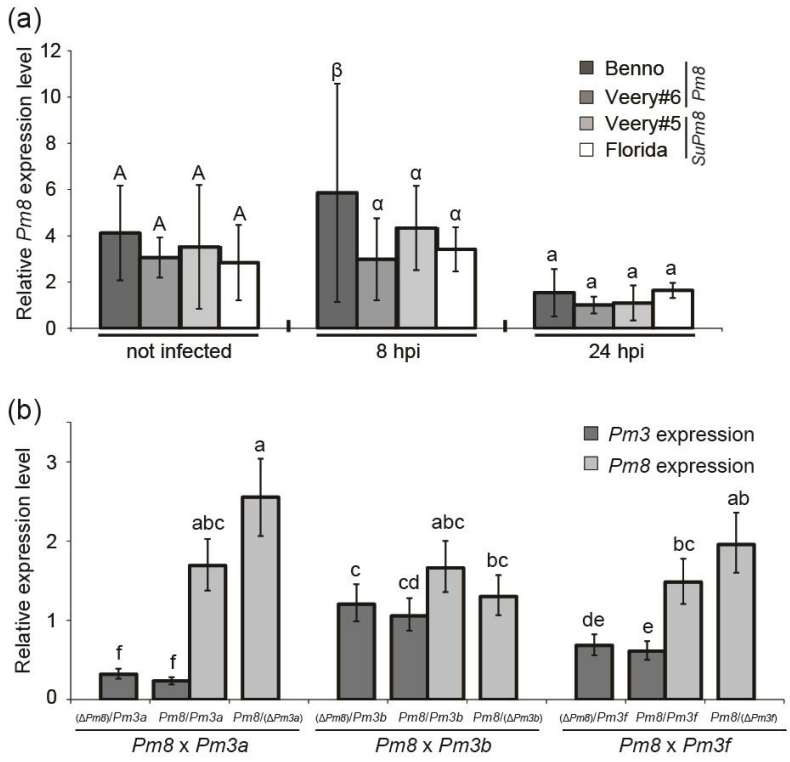

Figure 4. *Pm8* is expressed in suppressed *Pm8* lines to an equivalent level compared to non-suppressed lines.

(a) The relative expression level of *Pm8* was determined in *Pm8* lines Benno and Veery#6 and in the suppressed lines Veery#5 and Florida by reverse transcription, quantitative real-time PCR (RT-qPCR). Three biological replicates per line were analyzed from non-infected plants as well as plants eight and 24 hours after infection with *Bgt* isolate 07230, respectively (8 hpi; 24 hpi). The 95% confidence intervals are plotted and different letters on top of the bars denote a significant difference in expression level within each treatment / timepoint (Tukey's honestly significant difference test, α=0.050).

(b) Relative expression levels of *Pm3* and *Pm8* in transgenic lines were measured by RT-qPCR in three pooled first leaves of 10 day-old plants. Means of three biological replicates are shown relative to the *Pm3* expression level of one *Pm8*/*Pm3b* replicate used for normalising *Pm3* and *Pm8* expression levels. *Pm8* as well as *Pm3* were expressed to an equivalent level in the double-homozygous lines compared to their corresponding sister lines. The 95% confidence intervals are plotted and different letters on top of the bars denote a significant difference in expression level within each treatment / timepoint (Tukey's honestly significant difference test, α=0.050).

Since *Pm8* suppression is not caused by transcriptional gene silencing we checked if the suppression phenotype correlates with altered PM8 protein levels. The HA-epitope tag fused to the *Pm3* alleles and the myc tag fused to *Pm8* allowed specific detection of the corresponding proteins in the transgenic crosses. Equal amounts of total leaf protein extracts were loaded on protein gels for immunoblot analysis. Similar band intensities were detected for PM8 and PM3 proteins when the double-homozygous lines were compared to the corresponding sister lines (Figure 5). Therefore, suppression of *Pm8* does not correlate with lower protein amounts in the suppressed lines compared to the sister lines. Thus, suppression must occur at the post-translational level by a yet unknown mechanism.

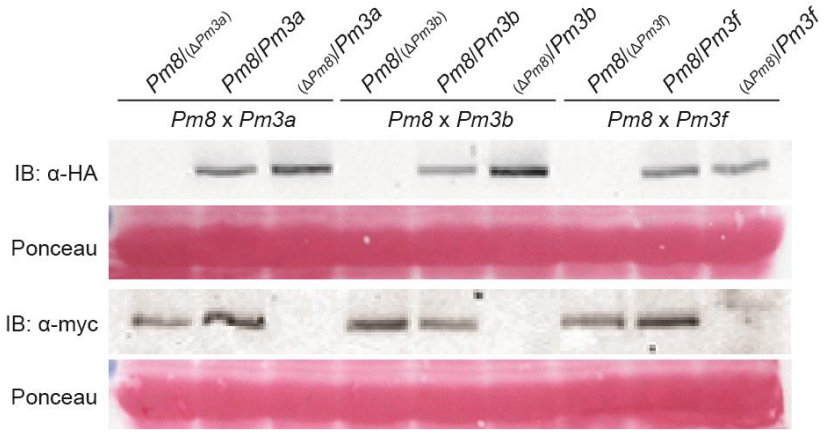

Figure 5. Similar PM8 and PM3 protein levels are present in double-homozygous compared to homozygous sister lines.

Immunoblot (IB) analysis detected similar amounts of HA-tagged PM3 proteins and myc-tagged PM8 proteins in double-homozygous lines (*Pm8*/*Pm3*) compared to their corresponding sister lines. Concentrations of total protein in leaf extracts of three pooled first leaves of 10-day-old plants were determined by Bradford protein analysis. The same protein amounts were loaded on protein gels with Ponceau-stained Rubisco large subunit (Ponceau) used as the loading control.

4.3.5 *Pm8*-mediated cell death in *Nicotiana benthamiana* is suppressed by *Pm3*

A possible post-translational suppression mechanism of *Pm8* could be based on interactions between the PM8 and PM3 proteins. To test this hypothesis, we analysed if PM8 and PM3 proteins interact with each other in a co-immunoprecipitation (co-IP) assay. However, the myc-tagged PM8 protein in transgenic wheat extracts could not be detected under this assay conditions. Therefore, we used the *Nicotiana benthamiana* system for which it was previously shown that an autoactive version of $PM8_{myc}$ ($PM8^{HR}$), which is mutated in the MHD motif, is able to mediate cell death (Stirnweis *et al.* 2014b). The $Pm8^{HR}$ construct was transiently expressed via *Agrobacterium tumefaciens* infiltration in leaf cells. In close proximity a non autoactive $Pm3CS_{HA}$ or $Pm3b_{HA}$ gene was infiltrated so that its infiltration spots partially overlapped with the $Pm8^{HR}$ infiltration spot. Indeed, $PM8_{myc}$-mediated cell death was suppressed by $Pm3CS_{HA}$ and $Pm3b_{HA}$ in the overlapping region (Figure 6a and 6b). In contrast, a GUS (*uidA*) control and a *Pm3* homolog, which is expressed in wheat and located on chromosome 1B (*Pm3*-$1B_{HA}$) (Hurni *et al*., 2013; GenBank KF572031), did not suppress *Pm8*-mediated cell death (Figure 6a and 6b). Thus, $Pm3CS_{HA}$ and $Pm3b_{HA}$ suppressed *Pm8*-mediated cell death in *N. benthamiana* leaves, reproducing the observation that *Pm3* is the suppressing gene of *Pm8* in wheat.

Suppression of *Pm8*-mediated cell death could be due to protein interactions of PM8 with PM3. To test for such interactions, we co-infiltrated a non-autoactive $Pm8_{myc}$ gene with $Pm3CS_{HA}$ or $Pm3b_{HA}$ into *N. benthamiana* leaf cells. Using anti-HA-tag magnetic beads the myc-tagged PM8 protein co-precipitated with $PM3CS_{HA}$ and $PM3B_{HA}$ (Figure 6c), suggesting an interaction between PM8 and PM3 proteins. $PM8_{myc}$ did not co-precipitate when co-infiltrated with the GUS_{HA} construct. In contrast, PM8 interacted with the *Pm3* homoeolog *Pm3*-$1B_{HA}$ (Hurni *et al.* 2013). As *Pm3*-$1B_{HA}$ does not

suppress *Pm8*$_{myc}$, we conclude that protein interaction *per se* is not sufficient for cell death suppression in *N. benthamiana*.

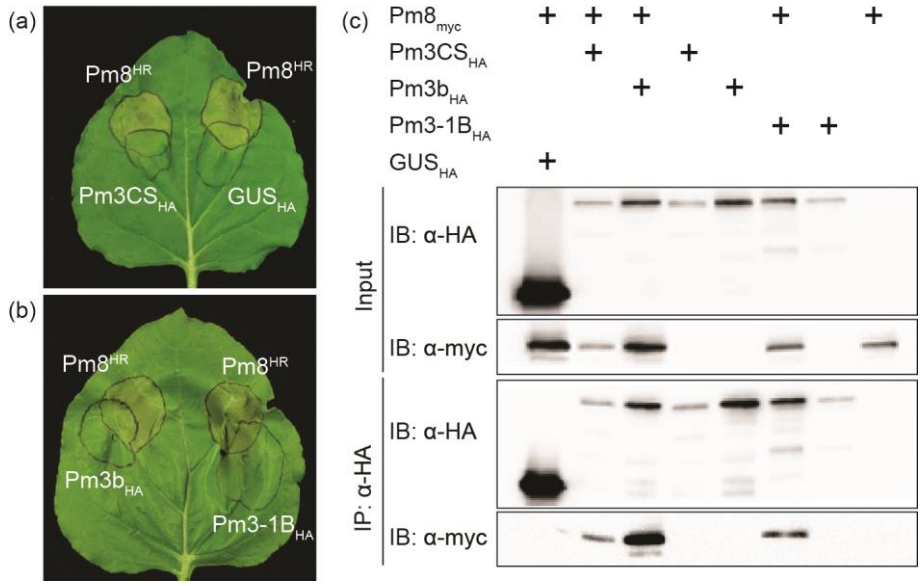

Figure 6. PM3CS and PM3B suppress *Pm8*-mediated cell death in *N. benthamiana* and interact with PM8.

(a,b) The *Pm8* gene mutated in the MHD motif (Pm8^{HR}) mediates cell death when infiltrated in *N. benthamiana*. No cell death was observed in overlapping infiltration areas with *Pm3CS*$_{HA}$ (a) and *Pm3b*$_{HA}$ (b) 48 hours after infiltration. In contrast, GUS (*uidA*) and *Pm3*-1B$_{HA}$ did not suppress *Pm8*^{HR}-mediated cell death.

(c) The myc tagged PM8 protein associated with immunoprecipitated PM3CS$_{HA}$, PM3B$_{HA}$ and PM3-1B$_{HA}$ protein but did not associate with GUS$_{HA}$. Total protein was extracted from three pooled *N. benthamiana* leafs 48 hours after co-infiltration of the constructs. Immunoblots (IB) before (Input) and after immunoprecipitation (IP) with anti-HA-tag magnetic beads are shown.

4.4 Discussion

Suppression of *R* genes has frequently been observed during the process of plant breeding but remained a phenomenon not understood at the molecular level. Here we show that suppression of the rye-derived *Pm8* gene in wheat is an excellent system to unravel the molecular mechanism of *R* gene interference. Suppression of *Pm8* has previously been described in diverse germplasm (Hanusova *et al.* 1996, Hao *et al.* 2012, McIntosh *et al.* 2011, Ren *et al.* 1997, Ren *et al.* 1996, Zeller and Hsam 1996), but the presence of the *Pm8* gene in the absence of a resistance reaction could only be inferred from the phenotypic analysis of rust resistance mediated by genes genetically linked to *Pm8* on the 1BL.1RS chromosome translocation derived from rye cultivar 'Petkus' (Ren *et al.* 1996). Based on the recent cloning of *Pm8* we show here that *Pm8*-suppressed lines carry an intact and expressed *Pm8* gene.

The *Pm8* suppressor gene was earlier genetically mapped to the *Pm3* locus (McIntosh *et al.* 2011). We found that the *Pm3* gene itself is sufficient to suppress *Pm8*-mediated resistance. Both susceptible (*Pm3CS* and *Pm3_8152)* as well as resistant alleles (*Pm3a*, *Pm3b* and *Pm3f)* suppressed *Pm8*. Therefore, it is likely that all 17 known resistant and the 37 susceptible *Pm3* alleles (Bhullar *et al.* 2010) can suppress *Pm8* when combined with this gene in the same wheat cultivar, and the suppression must be caused by a molecular property present in all alleles. As the sequences of *Pm3* alleles are all very similar (Bhullar *et al.* 2010), comparative analysis cannot reveal which part of the protein might be responsible for suppression. It was recently found that different *Pm3* alleles negatively interfere with each other in hybrid F1 plants as well as transgenic combinations (Stirnweis *et al*., 2014a, accompanying manuscript). There, it was found that the N-terminal half of the LRR domain is crucial for suppression activity. Assuming a similar molecular mechanism for *Pm8* suppression, the same region of the PM3 protein might be responsible for suppression.

The strength of *Pm8* suppression (none, weak or strong) depended on the specific transgenic *Pm3* allele combined with *Pm8* and the particular *Pm8* avirulent powdery mildew isolate used for infection. Complete suppression occurred for the *Pm8*/*Pm3b* combination with *Bgt* isolate AK3-11, whereas the combination with *Pm3a* and *Pm3f* showed only partial *Pm8* suppression. Similarly, very strong suppression was observed with the *Pm3f* combination using isolate C3-1 whereas the *Pm3a* combination showed a lower suppression with this isolate. This correlated well with the expression levels of the three *Pm3* alleles. While the ratio of the *Pm3b* to *Pm8* expression was 1:1, it was lower for *Pm3f* (1:3) and *Pm3a* (1:8). Therefore, the high *Pm8* expression compared to *Pm3a* and *Pm3f* might lead to an excess of PM8 protein which would then be able to partially overcome suppression and mediate some resistance. Similarly, a quantitative, dosage-dependent effect was found for the oat crown rust resistance gene *Pc-62*. There, the degree of suppression decreased by increasing *Pc-62* gene-dosage relative to the dosage of the suppressor locus (Wilson and McMullen 1997). However, protein amounts of PM3 alleles are very similar as shown in Figure 5. Therefore, the observed quantitative suppression of *Pm8* in the transgenic crosses could be due to differences in relative affinity of the homo- and heteromeric protein complexes in dependence of the avirulence gene and/or *Pm3* allele and/or the ability of the different complexes to function cooperatively.

Similar PM8 and PM3 protein levels in the double-homozygous transgenic lines compared to the sister lines indicated a post-translational suppression mechanism for *Pm8.* This was further supported by co-immunoprecipitation experiments that showed protein interactions of PM8 with PM3. Several plant NB-LRR proteins were shown to form homomeric complexes. For example, domains of the flax TIR-NB-LRR protein encoded by the *L6* resistance gene (Bernoux *et al.* 2011) or the barley CC-NB-LRR encoded by *Mla* (Maekawa *et al.* 2011a) were shown to form homodimers. The

formation of heteromeric complexes of NB-LRR proteins derived from orthologous genes or alleles, as shown here and in our companion publication (Stirnweis *et al*., 2014a, accompanying manuscript) might have a negative impact on protein complex function. This would then result in reduced or absent signal transduction after pathogen recognition. A dominant negative effect on resistance function has also been described for the tomato *Cf-9* receptor-like resistance gene (Barker *et al.* 2006). Wild type *Cf-9* activity was suppressed in a dominant-negative way by a truncated *Cf-9* gene, presumably by disturbance of signal transduction due to protein interference. Similarly, the wild type *N* tobacco mosaic virus resistance gene showed a dominant negative effect in the presence of a mutated *N* allele with TIR deletions or point mutations in the NBS domain (Dinesh-Kumar *et al.* 2000). Recently, it was found for the resistance protein pair RPS4/RRS1 that heterodimerization of their TIR domains in the absence of the pathogen leads to suppression of host defence signalling. Upon effector recognition conformational changes might lead to the signalling competent RPS4 TIR homodimer (Nishimura and Dangl 2014, Williams *et al.* 2014). Our proposed model where the interaction of PM8 and PM3 leads to the formation of a heteromeric protein complex which is not competent for signalling *Pm8* resistance can also explain earlier findings on resistance gene suppression in plant breeding. For example, interference by orthologous or other closely related proteins might be the cause of suppression of leaf rust resistance gene *Lr23* located on chromosome 2BS of wheat. Its suppressor was genetically mapped to the homeologous locus on chromosome arm 2DS (Nelson *et al.* 1997). Thus, we propose that the suppression mechanism observed for *Pm8* might be similar for other resistance genes and that resistance gene suppression in polyploid wheat might be caused by the presence of orthologous genes on the homoeologous chromosomes in general.

It is likely that certain avirulence gene products interact with the heteromeric protein complexes, resulting in the observed race-specificity for *Pm8* suppression in the lines combining transgenes. However, suppression of *Pm8* does not depend on factors from the pathogen, as it also occurred in infiltration experiments in *N. benthamiana*, in the absence of the pathogen. Isolate specificity of suppression was also described earlier for *R* genes against late blight in potato (Ordoñez *et al.* 1997) and against stripe rust (Chen *et al.* 2013), again suggesting similar processes at the molecular level.

Accessions of wild wheats as well as wheat relatives are good sources for disease resistance genes. However, the existence of resistance suppressors complicates the use of wild relatives for resistance breeding in bread wheat. Thus, it would be useful for breeders if suppression could be predicted as this might avoid costly and lengthy introgression projects that can take many years. Increasing knowledge on plant genomes, including the large wheat and barley genomes, will allow future prediction of potential problems in resistance gene introgression once the sequence of the gene to be introgressed is known. For example, recipient genotypes with potential suppressors could be avoided completely. Furthermore suppressor genes could be mutated or replaced by non-suppressing genes using new breeding technologies for targeted genome editing. Mutagenesis also has the potential to unlock resistance gene diversity in many other plant lines. Several studies have found that mutant screens resulted in resistant plants (Boyd and Minchin 2001), an observation which can easily be explained by the mutation of suppressor genes. The identification of additional suppressor genes in crop plants and the molecular characterization of the protein sequences involved in dominant negative interference will allow the development of strategies to minimize such negative effects in future resistance breeding which is expected to depend strongly on the use of crop relatives.

4.5 Experimental Procedures

4.5.1 Amplification of *Pm8* and *Pm3* by nested PCR

The coding region of *Pm8* was amplified in a two-step nested PCR as described in Hurni *et al*. (2013). PCR amplification of *Pm3* alleles was essentially performed as described in Srichumpa *et al*. (2005). Instead of primer N3'SP3R primer SuB24 (5'-GTGCAACAATCAGGGATCAG-3') was used, and the iProof high-fidelity DNA polymerase (1725300; Bio-Rad, http://www.bio-rad.com/) was employed. The sequence of the primer SI-1 is 5'-TATATAGTCGACGCTTCAGCTCCGGCAGGCCTG-3'.

4.5.2 Analysis of the *Pm8* translocation lines

The *Pm8* translocation lines (for references see http://www.rye-gene-map.de/rye-introgression/), their infection with powdery mildew and plant growth conditions were described previously (Hurni *et al.* 2013).

4.5.3 Analysis of the wheat cross Chinese Spring x Kavkaz/4*Federation

The wheat line Chinese Spring carrying the *Pm3* allele *Pm3CS* was crossed with the *Pm8* carrying translocation line Kavkaz/4*Federation. In the F_2 generation, 46 plants were analysed with a *Pm3*-specific PCR marker amplifying a 922 bp fragment in the 5'UTR of *Pm3* and a 1.1 kb fragment from the *Pm3*-1B gene on chromosome 1B (Hurni *et al.* 2013, Tommasini *et al.* 2006). PCR was performed with the forward primer UP3B (5'- TGGTTGCACAGACAATCC-3') and reverse primer UP1A (5'-GAAACCCGGCATAAGGAG-3') in a total volume of 25 µl with 0.05 units/µl Taq DNA polymerase (D1806; Sigma-Aldrich, http://www.sigmaaldrich.com/). In addition, plants were analysed with the *Pm8*-specific PCR marker *sfr43(Pm8)* (Hurni *et al.* 2013). Based on this pre-selection, F_3 plants were analysed with the same markers to identify

segregants that were either homozygous for *Pm3CS* or *Pm8* or homozygous for both genes (double-homozygous). The homozygous and double-homozygous status of the selected lines was confirmed by PCR markers in the F_4 generation. Powdery mildew infection tests were performed on F_4 seedlings with *Pm8*-avirulent *Bgt* isolate 07230 and *Pm8*-virulent isolate 07250 in leaf segment infection tests as described by Hurni *et al*. (2013). In total, 24 F4 individuals per genotype were tested in three independent powdery mildew infection tests with isolate 07230. With isolate 07250, in total 9 F4 individuals per genotype were tested in two independent powdery mildew infection tests.

4.5.4 Analysis of transgenic crosses *Pm3* x *Pm8*

The *Pm3* transgenic lines Pm3a#1, Pm3f#1 (Brunner *et al.* 2012) and $Pm3b_{HA}$ (Stirnweis *et al*., 2014a, accompanying manuscript) were each crossed with the *Pm8* transgenic line Pm8#59 (Hurni *et al.* 2013). Segregant analysis for selection of homozygous and double-homozygous lines was performed by PCR markers in the F_3 generation. For *Pm8* detection, the forward primer UP81A (5'-AAGAAGCTCCCTAGATGC-3') in the 3' sequence of the *Pm8* gene and the reverse primer dst006 (5'-ACGGATCCTCACAAATCT-3') located on the myc epitope tag sequence amplifying a 390 bp fragment was used. For *Pm3* detection, the forward primer sbi342 (5'-TGGGCAGCATCAAACGC-3') in the 3' sequence of the *Pm3* gene and the reverse primer sbi143 (5'-CAAGACCGGCAACAGGATTC-3') on the nopaline synthase terminator (nos) sequence amplifying a 430 bp fragment was used. PCRs were performed in total volumes of 25 µl with 0.05 units/µl Taq DNA polymerase (D1806; Sigma-Aldrich) and an annealing temperature of 58°C and 62°C, respectively. Powdery mildew infection tests were performed on the first leaves of 10-day-old F_4 seedlings of *Pm3* and *Pm8* homozygous sister lines as well as the double-homozygous lines. Two infection tests were performed with *Bgt* isolates C3-1, 10004, 98229 and

10004 using eight to ten seedlings per line and three infection tests with *Bgt* isolates AK3-11, 10001, 07250 and 97011 using 15 to 16 seedlings per line. The mean infected leaf areas from these experiments are reported in Figure S2. Isolates AK3-11 and C3-1 were obtained from USDA, North Carolina State University, USA, whereas the other isolates were collected in Switzerland and are maintained in our powdery mildew collection. Detached leaves infected with powdery mildew were kept on 0.5% phytoagar (supplemented with 30 ppm benzimidazol) plates at 20°C, 80% relative humidity and 16 h light. Powdery mildew infection and scoring were as previously described (Kaur *et al.* 2008, Winzeler *et al.* 1991).

4.5.5 Single-cell transient expression assay

The plasmid construct pGY1-*Pm3CS* (Yahiaoui *et al.* 2006) or the empty vector control plasmid pGY1, were biolistically co-transferred together with the reporter plasmid pUbi-GUS into wheat seedlings and the results were evaluated as described earlier (Hurni *et al.* 2013).

4.5.6 Quantitative real-time PCR for measuring *Pm8* and *Pm3* expression levels

Expression of *Pm8* and *Pm3* was quantified as essentially described earlier (Hurni *et al.* 2013). In short, for each wheat line, technical triplicates of three biological replicates were analyzed. Each biological replicate consisted of three pooled first leaves of 10-day-old seedlings. Two stably expressed reference genes were used for nomalizing the expression data (Table S3 and S4). For a more detailed description see Methods S1.

4.5.7 Protein detection

Total protein was extracted from three pooled primary wheat leaves and analyzed as essentially described earlier (Brunner *et al.* 2012) but using a different extraction buffer (50 mM Tris/HCl pH8, 25 mM sucrose, 5 mM EDTA, 5 mM DTT, 1x complete protease

inhibitor cocktail (04693159001; Roche, http://www.roche-applied-science.com/)). Eighty µg protein was loaded and anti c-myc primary antibody (GTX10910; GeneTex, http://www.genetex.com/) and secondary antibody were used in a 1 : 3000 dilution. For detection of peroxidase activity, the Chemidoc XRS system (Bio-Rad) was used.

4.5.8 Agroinfiltrations and co-immunoprecipitation

All plasmid constructs infiltrated into *N. benthamiana* contained genomic DNA of the respective genes in the binary vector pIPKb004 (Himmelbach *et al.* 2007) and were produced according to (Stirnweis *et al.* 2014b). For overlapping infiltrations, *A. tumefaciens* containing the $Pm8^{HR}$ construct were infiltrated at least 2 h before infiltration with $Pm3CS_{HA}$, $Pm3b_{HA}$, $Pm3\text{-}1B_{HA}$ or GUS_{HA}. Co-immunoprecipitation was performed according to (Stirnweis *et al.* 2014b), except that we used 30 µl of anti-HA-tag magnetic beads (M132-9; MBL, http://www.mblintl.com/) and antibodies described above for protein detection. Single constructs were co-infiltrated, with an untagged GUS construct to keep a 1:1:1 infiltration ratio (Stirnweis *et al.* 2014b).

4.6 Acknowledgements

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4.7 Supporting Information

Methods S1. Supplementary experimental procedures.

RT-qPCR analysis for detection of *Pm8* and *Pm3* expression

Expression of *Pm8* and *Pm3* was quantified in a reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) assay using a CFX96 Real-Time System C1000TM Thermal cycler (Bio-Rad, http://www.bio-rad.com/) and for the Chinese Spring x Kavkaz/4*Federation cross an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, http://www.lifetechnologies.com/). The translocation lines (Figure 4a) and the lines of the cross Chinese Spring x Kavkaz/4*Federation (Figure S3) were grown at 17°C, 70% relative humidity and 16 h light. Ten-day-old plants were then either infected with the *Pm8* avirulent powdery mildew isolate 07230 or left without infection and covered with gas permeable covers. For the translocation lines leaf samples were taken after eight hours from infected and non infected plants and after 24 hours from infected plants. For lines of cross Chinese Spring x Kavkaz/4*Federation leaf samples were taken after 24 hours from infected and non-infected plants. Leaf samples were taken from ten-day-old transgenic plants (Figure 4b) which were grown at cycles of 16 h at 20°C with light and 8 h at 16°C in the dark at a constant relative humidity of 70%. RNA extraction and first-strand cDNA synthesis of 1 µg RNA were performed as described by Hurni *et al*. (2013). RT-qPCR primers and probes for the targets *Pm8* and *Pm3* and the reference genes were adopted from earlier studies (Brunner *et al.* 2011, Hurni *et al.* 2013, Travella *et al.* 2006) and are described in Tables S3 and S4.

For the translocation lines and transgenic crosses (Figure 4), RT-qPCR was performed with 4 µl of tenfold diluted cDNA, forward and reverse primers according to Table S3 and 5 µl of KAPA PROBE FAST qPCR Master Mix (KK4701; Kapa Biosystems, http://www.kapabiosystems.com/) for *Pm8* and *Pm3* and KAPA SYBR® FAST qPCR

Master Mix (KK4601; Kapa Biosystems) for *ADP* and *TA.6863* in a total reaction volume of 10 µl. Thermocycling conditions were 95°C for 20 s, followed by 40 cyles of 95°C for 3 s, then 60°C for 20 s. Specificities of the amplicons were checked by examination of dissociation curves with CFX Manager 3.1 Software (Bio-Rad) for the two SYBR® green-based targets *ADP* and *TA.6863.* RT-minus controls were checked with the reference gene target ADP. All RT-minus controls had quantification cycle (Cq) values of at least 12 cycles above the corresponding RT-plus samples, thus DNA contamination was irrelevant (>0.025%). The primer-probe combination for *Pm8* showed no fluorophor signal on cDNA of lines homozygous for *Pm3* and vice versa, therefore RT-qPCR assay specificities for the orthologous genes *Pm8* and *Pm3* were confirmed. Sensitivities of *Pm8* and *Pm3* qPCR assays were compared on the basis of plasmid DNA containing either the cloned *Pm8* or the *Pm3* RT-qPCR amplicon. At identical plasmid concentrations equal Cq values were obtained when the same threshold values were applied for both targets, hence both assays had the same sensitivity and expression levels were directly comparable among *Pm8* and *Pm3*. Relative quantities were calculated and normalized to the two reference genes *ADP* and TA.6863 (CNRQ) using the program qbase+ V 2.6 (Biogazelle, http://www.biogazelle.com/). Target-specific amplification efficiencies are given in Table S3. For a description of efficiency calculation and RT-qPCR set up see Risk *et al.* (2012).

RT-qPCR was performed with the lines from the cross Chinese Spring x Kavkaz/4*Federation with 4 µl of tenfold diluted cDNA, forward and reverse primers according to Tables S4 and 6 µl of TaqMan® Fast Universal PCR Master Mix (4352046; Life Technologies) for *Pm8* and *Pm3* and *CDCP* Fast SYBR® Green Master Mix (4385612; Life Technologies) for *GAPDH* in total reaction volumes of 16 µl. Thermocycling conditions were 95°C for 20 s, followed by 40 cyles of 95°C for 4 s, then

60°C for 30 s. For the SYBR® green based target *GAPDH* specificity of the amplicon was checked by examination of dissociation curves with 7500 Software v2.0.6 (Life Technologies). RT-minus controls were checked with the target *GAPDH*. All RT-minus controls had quantification cycle (Cq) values of at least 12 cycles above the corresponding RT-plus samples, thus DNA contamination was irrelevant (>0.025%). Relative quantities were calculated and normalized to the reference genes *GAPDH* and *CDCP* (CNRQ) using the program qbase+ V 2.6 (Biogazelle). Target-specific amplification efficiencies are given in Table S4.

Data analysis was performed using the statistical package JMP version 9.0 (SAS Institute, http://www.sas.com/). Tukey's honestly significant difference test for the transgenic lines (Figure 4b) was done on log10 transformed expression values. In Figure 4b, the untransformed means are given and back-transformed 95% confidence intervals are indicated.

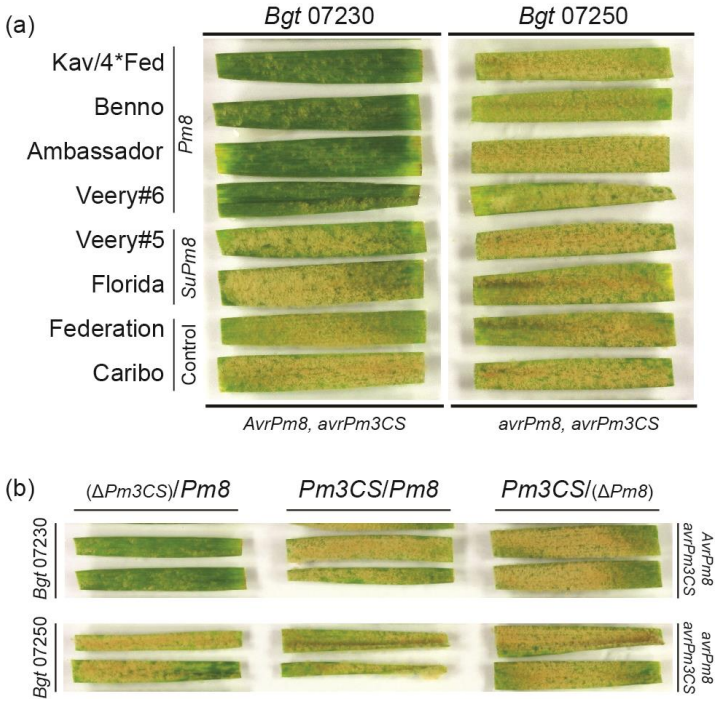

Figure S1. The *Pm8* wheat lines Veery#5, Florida and *Pm3CS*/*Pm8* are susceptible to *Pm8*-avirulent (*AvrPm8*) *Bgt* isolate 07230.

(a) The four wheat lines Kavkaz/4*Federation (Kav/4*Fed), Benno, Ambassador and Veery#6 carrying *Pm8* but no *Pm3* allele, as determined by PCR, were resistant to *Bgt* isolate 07230 which is avirulent to *Pm8*. In contrast, the two lines Veery#5 and Florida carrying in addition to *Pm8* the *Pm3* alleles *Pm3_8152* and *Pm3CS*, respectively, were highly susceptible. As susceptible control lines Federation (recurrent parent of Kavkaz/4*Federation) and Caribo (in the pedigree of Florida; carries the *Pm3CS* allele) were used.

(b) The double-homozygous segregant *Pm3CS*/*Pm8* from a cross between Chinese Spring (*Pm3CS*) and Kavkaz/4*Federation (*Pm8*) was susceptible to *Bgt* isolate 07230. In contrast, the homozygous *Pm8* segregant was resistant. All lines were highly susceptible to isolate 07250 which is virulent to *Pm8* (*avrPm8*).

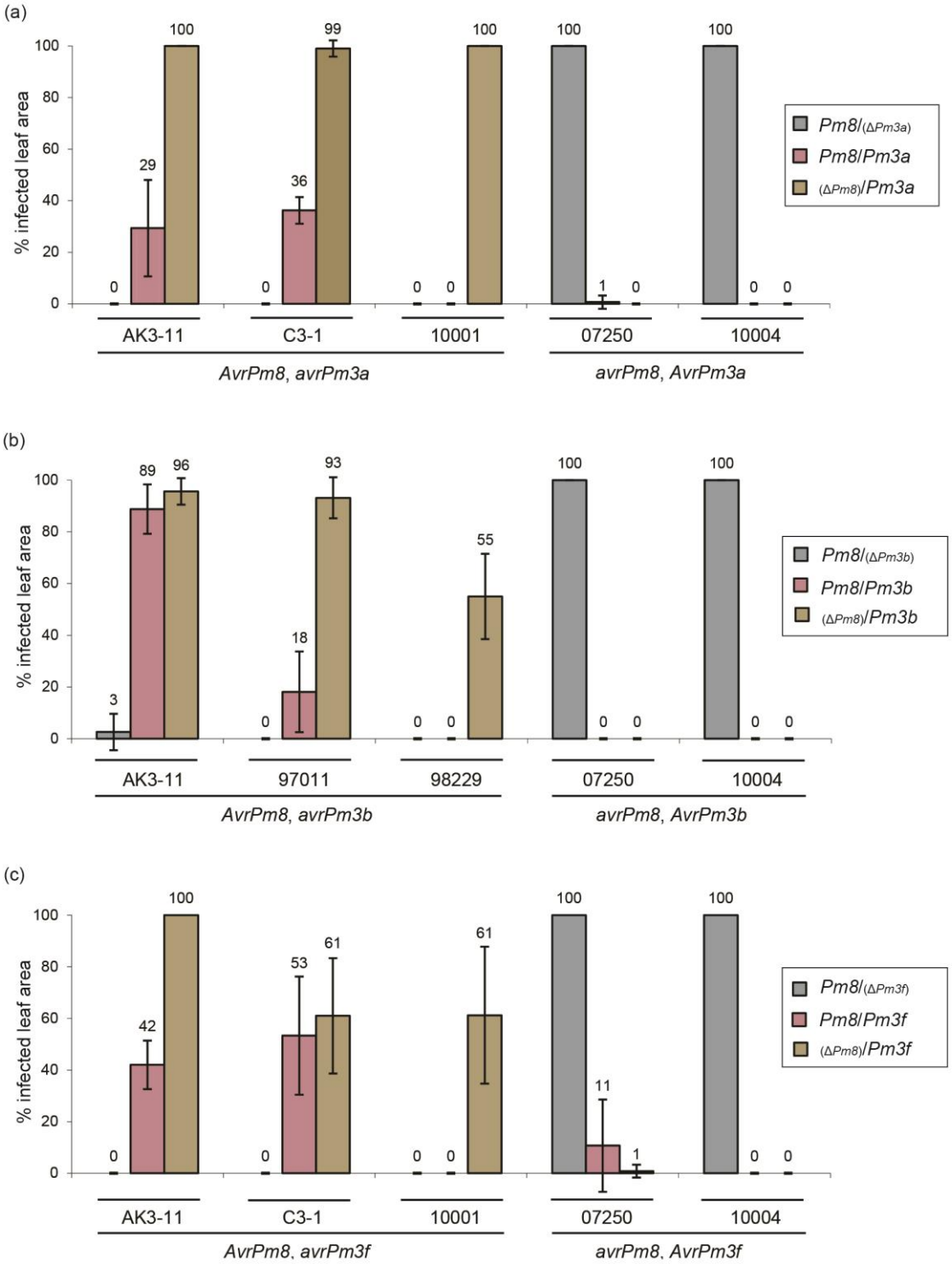

Figure S2. *Pm8*-mediated resistance is suppressed in double-homozygous segregants carrying both the *Pm8* transgene and a transgenic allele of *Pm3*.

(a-c) The double-homozygous lines *Pm8*/*Pm3a* (a) and *Pm8*/*Pm3f* (c) had mean infected leaf areas of 29-53% when infected with isolates AK3-11 and C3-1, but 0% with isolate 10001. In

contrast, the *Pm8* sister lines *Pm8*/(Δ*Pm3a*) and *Pm8*/(Δ*Pm3f*) were completely resistant to all three isolates (0%). Line *Pm8*/*Pm3b* (b) was nearly as susceptible as the sister line (Δ*Pm8*)/*Pm3b* (89% versus 96%) to isolate AK3-11 but only slightly susceptible to isolate 97011 (18%) and fully resistant to isolate 98229 (0%). The *Pm8*/(Δ*Pm3b*) sister line was resistant to all three isolates (< 3%). All three double-homozygous lines showed *Pm3*-mediated resistance to isolates 07250 and 10004 as well as the *Pm3* sister lines (Δ*Pm8*)/*Pm3a*, (Δ*Pm8*)/*Pm3b and* (Δ*Pm8*)/*Pm3f* (< 1%). However, the *Pm8*/*Pm3f* line was slightly susceptible to isolate 07250 (11%). The first leaves of 10-day-old F_4 seedlings were infected with the *Bgt* isolates and kept at 20°C and 80% relative humidity for eight days until the percentage of infected leaf area was determined visually. The mean percentages of infected leaf area of 8 to 10 seedlings per line from two infection tests are shown for *Bgt* isolates C3-1, 10004, 98229 and 10004. For the *Bgt* isolates AK3-11, 10001, 07250 and 97011 mean percentages of infected leaf area of three infection test with 15 to 16 seedlings per line are shown. Error bars indicate standard deviations. The presence (*Avr*) or absence (*avr*) of the relevant avirulence gene is indicated below the *Bgt* isolate name.

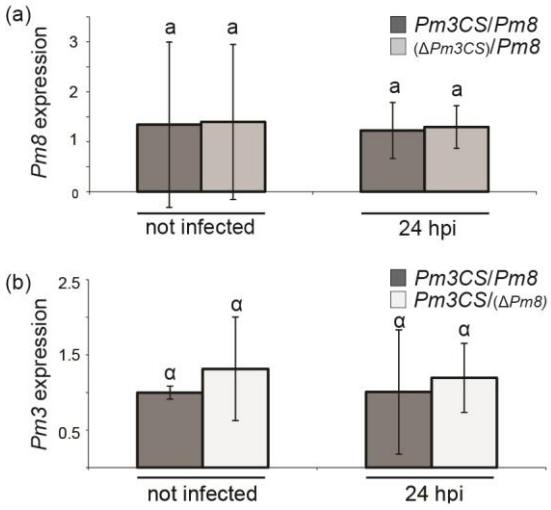

Figure S3. *Pm8* and *Pm3CS* are expressed in the double-homozygous line *Pm3CS*/*Pm8*.

(a) Expression of *Pm8* was measured by RT-qPCR of non-infected plants and plants 24 hours post infection (24 hpi) with the *Pm8*-avirulent *Bgt* isolate 07230. The expression level of *Pm8* in the double-homozygous line *Pm3CS*/*Pm8* was not significantly different from the expression level in the homozygous *Pm8* sister line ((Δ*Pm3CS*)/*Pm8*) without and with infection. Plants were obtained by crossing line Kavkaz/4*Federation with Chinese Spring and selecting segregants in the F_3 generation. Three biological replicates were analyzed per line and timepoint. The 95% confidence intervals are plotted and identical letters on top of the bars denote no significant difference in the *Pm8* expression level (Tukey's honestly significant difference test, α=0.050).

(b) The expression level of *Pm3CS* in the line *Pm3CS*/*Pm8* was equivalent to the expression level in the *Pm3CS* homozygous sister line *Pm3CS*/(Δ*Pm8*) without infection and 24 hours post infection. Three biological replicates were analyzed per line and timepoint. The 95% confidence intervals are plotted and identical letters on top of the bars denote no significant difference in the *Pm8* expression level (Tukey's honestly significant difference test, α=0.050).

Table S1. Powdery mildew isolates used in seedling infection tests as well as transient expression assays.

Bgt Isolate	Avirulence[a]	Virulence[b]
AK3-11	*AvrPm8*	*avrPm3a*, *avrPm3b*, *avrPm3f*
C3-1	*AvrPm8*	*avrPm3a*, *avrPm3f*
97011	*AvrPm8*	*avrPm3b*
98229	*AvrPm8*	*avrPm3b*
07230	*AvrPm8*	*avrPm3CS*[c]
07250	*AvrPm3a*, *AvrPm3b*, *AvrPm3f*	*avrPm8*, *avrPm3CS*
10001	*AvrPm8*	*avrPm3a*, *avrPm3f*
10004	*AvrPm3a*, *AvrPm3b*, *AvrPm3f*	*avrPm8*

[a] For each *Bgt* isolate, the avirulence (*Avr*) genes relevant for this work are given.
[b] For each *Bgt* isolate, the absent avirulence (*avr*) genes relevant for this work are given.
[c] *Pm3CS* is a susceptible *Pm3* allele (Yahiaoui *et al.* 2006).

Table S2. Haustorium indices (%) with *Bgt* isolate 07230 reported in Figure 1.

		HI (%) 07230				
Construct	Line	Rep 1	Rep 2	Rep 3	Rep 4	Mean ± SD
Empty vector	Federation	61	68	73		67 ± 6
Pm3CS	Federation	64	62	73		66 ± 6
Empty vector	Kav/4*Fed	27	25	16		23 ± 6
Pm3CS	Kav/4*Fed	37	44	34		38 ± 5
Empty vector	Benno	13	29	12	13	17 ± 8
Pm3CS	Benno	29	42	34	34	35 ± 5
Empty vector	Ambassador	18	16	12	2	12 ± 7
Pm3CS	Ambassador	31	31	20	32	29 ± 6

Table S3. Primers and probes used for RT-qPCR assay in Figure 4.

Target gene (UniGene / Genbank)	Gene name	5'-3' Sequence; modifications	Primer/Probe concentration nM	PCR efficiency (E) r^2 of calibration curve Slope	Amplicon length bp	Reference
KF572030	*Pm8*	F CTGGGCAGCATCAAGGA R CCGCTCACGGACTAGCCTC Probe VIC-CCTGCTATAAAGCAACG-MGBNFQ	250 250 250	E= 98% r^2 = *0.987* Slope = -3.37	108	(Brunner *et al.* 2011, Hurni *et al.* 2013)
TA.31015	*Pm3*	F TGGGCAGCATCAAACGC R CCGCTCACGGACTAGCCTC Probe FAM-TGCCCGTTATGAAGTAA-MGBNFQ	250 250 250	E= 99% r^2 = *0.996* Slope = -3.35	107	(Brunner *et al.* 2011, Hurni *et al.* 2013)
TA.2291	*ADP*	F CTGGAGCACGAAGCTGCAG R CGAGTGCTGGAGCTTGCAGT	250 250	E= 100% r^2 = *0.999* Slope = -3.31	80	(Gimenez *et al.* 2011, Hurni *et al.* 2013)
TA.6863	*TA.6863*	F GCAGGGTCAGGAAGATATTGG R GAATCTGGCCTACGGTTGAT	250 250	E= 98% r^2 = *0.995* Slope = -3.38	105	(Hurni *et al.* 2013)

Table S4. Primers and probes used for RT-qPCR assay in Figure S3.

Target gene (UniGene / Genbank)	Gene name	5'-3' Sequence; modifications	Primer/Probe concentration nM	PCR efficiency (E) r^2 of calibration curve Slope	Amplicon length bp	Reference
KF572030	*Pm8*	F CTGGGCAGCATCAAGGA R CCGCTCACGGACTAGCCTC Probe VIC-CCTGCTATAAAGCAACG-MGBNFQ	250 250 250	E= 98% r^2 = *0.998* Slope = -3.36	108	(Brunner *et al.* 2011, Hurni *et al.* 2013)
TA.31015	*Pm3*	F TGGGCAGCATCAAACGC R CCGCTCACGGACTAGCCTC Probe FAM-TGCCCGTTATGAAGTAA-MGBNFQ	600 600 250	E= 94% r^2 = *1.000* Slope = -3.48	107	(Brunner *et al.* 2011, Hurni *et al.* 2013)
TA.54533	*GAPDH*	F TTAGACTTGCGAAGCCAGCA R AAATGCCCTTGAGGTTTCCC	600 600	E= 94% r^2 = *0.999* Slope = -3.49	81	(Travella *et al.* 2006)
TA.54227	*CDCP*	CAAATACGCCATCAGGGAGAA GCTTCAGGGTTGTCCTTCCTC Probe NED-CTCTCGATGTCCTTCTC-MGBNFQ	600 600 250	E= 97% r^2 = *0.993* Slope = -3.41	72	(Brunner *et al.* 2011, Paolacci *et al.* 2009)

5. General Discussion

5.1 Rapid and easy cloning of *R* gene orthologs is feasible by homology-based cloning

In the past, map-based cloning was used to identify a growing number of resistance genes from Triticeae species (Feuillet *et al.* 2012, Krattinger *et al.* 2009). For example the barley powdery mildew resistance gene *Mla* (Wei *et al.* 1999, Zhou *et al.* 2001), the wheat stem rust resistance genes *Sr33* and *Sr35* (Periyannan *et al.* 2013, Saintenac *et al.* 2013) and the wheat powdery mildew resistance gene *Pm3* (Yahiaoui *et al.* 2004) were cloned from barley and wheat. This approach has been very laborious and time-consuming, often taking more than a decade from the project start to the final cloning of the gene. This was mainly due to problems related to the large Triticeae genomes of 17Gb for wheat, 5Gb for barley and 8Gb for rye, their high number of repetitive sequences as well as underdeveloped genomic resources (Feuillet *et al.* 2012, Krattinger *et al.* 2009). Using a homology based approach, including allele mining, in total 54 alleles of *Pm3* (Bhullar *et al.* 2009, Bhullar *et al.* 2010, Kaur *et al.* 2008, Srichumpa *et al.* 2005), and a series of genes at the *Mla* locus (Seeholzer *et al.* 2010) were cloned. The purpose of the work in the second chapter of this thesis was to identify by homology-based cloning the powdery mildew resistance gene *Pm8* from rye, using a similar approach as it was used for the identification of the *Pm3* alleles. Since *Pm8* was mapped at the distal end of chromosome 1RS (Hulbert *et al.* 2001, Sandhu and Gill 2002), in the syntenic, gene-dense region of *Pm3* (wheat chromosome 1AS) (Yahiaoui *et al.* 2004), we hypothesized that *Pm8* and *Pm3* are orthologs. This was further supported by the identification of a common, *Pm3* homologous sequence in 1BL.1RS translocation lines as well as 'Petkus' rye lines by Southern blot analysis using a *Pm3* haplotype-specific probe derived from the 5' region of *Pm3*. Therefore, we designed primers highly conserved among the *Pm3* alleles, to identify the *Pm8* gene by

a homology-based cloning approach. We were able to amplify a *Pm3* homologous sequence from the 1BL.1RS translocation in wheat as well as from its rye donor 'Petkus' which we functionally and genetically identified to be *Pm8*. In the past, it has been shown that allele mining works very well for the identification of allelic genes but that it can be difficult to identify more distantly related sequences of functional *R* genes due to the large number of *R* genes which are present in plants (Sekine *et al.* 2012). Cloning of the functional *R* gene *Pm8* here shows that this is not necessarily true. Instead, it is feasible to clone orthologous *R* genes by a homology-based approach from such distantly related plants as wheat and rye, who diverged about 7 million years ago (Huang *et al.* 2002a, Huang *et al.* 2002b). Similarly, a homolog of barley *Mla* was cloned from wheat and found to be functional against wheat powdery mildew even though this gene was not mapped in wheat before (Jordan *et al.* 2011). Here, homology-based cloning proved to be a straightforward and fast way of cloning a major resistance gene for powdery mildew.

5.2 *Pm8*/*Pm3* homologs in other grass species: a source for new *R* genes?

An *in silico* search for *Pm8*/*Pm3* homologs in the genomes of grasses could be a promising approach for the identification of new functional resistance genes. Ongoing sequencing projects of Triticeae genomes, such as the wheat genome sequencing project (http://www.wheatgenome.org/), the available barley genome (Mayer *et al.* 2012) and sequences from the rye genome (Bartoš *et al.* 2008, Martis *et al.* 2013), are making this a realistic approach. This includes also resequencing projects which will allow access to intraspecific diversity present at resistance loci. The enormous amount of Triticeae sequences becoming available will also facilitate primer design for homology-based cloning and will accelerate the identification of new resistance gene

candidates. Since *Pm3* and *Pm8* kept their function as powdery mildew resistance genes over 7 million years, this approach is promising and it is likely to identify functional powdery mildew resistance gene homologs from other Triticeae species. Using this approach, we already identified *in silico* a *Pm3* homolog from the barley genome sequence, *HvPm3* (CAJX010064507.1). Whether this gene is able to confer powdery mildew resistance in wheat remains to be tested in a transient or transgenic assay. *Pm8* homologs or alleles may also be found in the rye genome itself. In the literature, a possible allele of *Pm8* was described, *Pm17*, present in wheat cultivar 'Amigo' on the 1AL.1RS translocation derived from the rye source 'Insave' (Heun *et al.* 1990, Hsam and Zeller 1997, Lowry *et al.* 1984). We identified by homology-based cloning a *Pm17* candidate gene and developed a specific PCR marker for it (our unpublished data). If we can confirm that the candidate gene is a functional resistance gene and identify it as *Pm17*, further studies will be possible. In an earlier study, the powdery mildew resistance segregation ratio of two mapping populations suggested that *Pm8* and *Pm17* are alleles (Hsam *et al.* 1995, Hsam and Zeller 1997). This could be further confirmed with the gene specific markers for *Pm8* (chapter 2) and *Pm17* (our unpublished data). For *Pm3*, the wheat ortholog of *Pm8*, 54 alleles were cloned (Bhullar *et al.* 2010). Therefore, more *Pm8* alleles could possibly be identified in diverse rye cultivars from broad geographical origin by homology-based cloning. In addition, *Pm8* homologs might be found in the rye genome, since we detected several homologous sequences by Southern blot analysis with a *Pm3* haplotype specific probe in 'Petkus' as well as 'Imperial' rye cultivars. It will be crucial in the future to have efficient and fast screening methods to identify functional resistance genes out of many identified homologous *R* gene sequences. Such genes could be introgressed into wheat cultivars or used in a transgenic approach to improve powdery mildew resistance in crops.

5.3 Broader race-specificity by combining *Pm3*/*Pm8* sequences in chimeric genes?

Pm3 alleles share more than 97% sequence identity, therefore there is limited sequence diversity (Bhullar *et al.* 2010, Yahiaoui *et al.* 2006). A much greater difference exists between *Pm3* and its ortholog *Pm8*. However, there are sequence blocks and domains which are highly conserved between *Pm8* and *Pm3*. Strikingly, we found the ARC2 subdomain to be conserved between the PM8, PM3A and PM3B proteins. This domain provides an extended resistance spectrum of *Pm3a* and *Pm3b* alleles compared to their narrow resistance spectrum partner alleles *Pm3f* and *Pm3c*, therefore contributing to a quantitatively higher powdery mildew resistance level (chapter 1) (Brunner *et al.* 2010). Interestingly, it was recently found that only two amino acids in this domain correlate with a fast and intense hypersensitive response (HR) in a *Nicotiana* transient-expression system, and can broaden the race-specificity of *Pm3f* in a single-cell transient expression assay in wheat (Stirnweis *et al.* 2014b). It was also shown for other allelic or paralogous resistance genes that polymorphisms in the TIR, CC, and NB domain can influence the resistance spectra (reviewed in Stirnweis *et al.* 2014b). Combining polymorphisms in the LRR domain of two *Pm3* alleles also led to an extended resistance spectrum and a single amino acid change converted the susceptible *Pm3CS* allele into a resistant allele (chapter 1) (Brunner *et al.* 2010, Yahiaoui *et al.* 2006). Highest diversity among *Pm3* alleles was found in the LRR domain. Similarly, we found high sequence diversity in the solvent-exposed residues of PM8 compared to PM3 (chapter 2). Using the high diversity of these amino acids in PM8 in combination with PM3 sequences might lead to a new and/or broader race spectrum and a quantitatively increased powdery mildew resistance mediated by artificially designed resistance genes. In contrast to the high diversity found between *Pm3* and *Pm8* in the LRR domain, the CC domain is highly conserved, sharing 96%

sequence identity. This correlates well with the complete conservation of this domain among the 31 *Pm3* alleles known from hexaploid wheat, and only three nucleotide polymorphisms in the 23 *Pm3* alleles from tetraploid wheat (Bhullar *et al.* 2010, Yahiaoui *et al.* 2009). Intergenic domain swap experiments between the CC domain of *Pm8* and the NB-LRR domain of *Pm3* could reveal the functional relevance of this domain for the resistance spectrum. Similar studies can be performed with the LRR domain or single amino acid changes to check whether interspecific sequence exchanges lead to functional R proteins with an altered, potentially expanded resistance spectrum. Three earlier studies have demonstrated that artificially improved genes can be made. Random mutagenesis in the LRR domain of the *Rx* gene (Farnham and Baulcombe 2006), amino acid substitutions in the NB and ARC1 subdomains of Rx (Harris *et al.* 2013), and the substitution of two amino acids in the ARC2 subdomain of PM3 (Stirnweis *et al.* 2014b) resulted in improved resistance genes.

5.4 Molecular dissection of resistance gene suppression

During synthetic wheat production it was observed that a disease resistance gene active in one of the parents was not functional in the hexaploid wheat or shows decreased activity (Assefa and Fehrmann 2004, Boyd 2005, Kema *et al.* 1995, Ma *et al.* 1995). This has in general been observed when genes from a lower ploidy genome were introduced into a higher one leading to a resistance dilution effect. The causative genes for low or absent resistance expression have been called suppressors or modifiers in the literature. There is a rich literature in classical genetics on the suppression of resistance genes in wheat in certain crosses, but little is known on its molecular basis (reviewed in chapter 1.4.4). In chapter 3, we analyzed the suppression mechanism of the wheat powdery mildew resistance gene *Pm8*. Alleles of the *Pm3* gene were identified as suppressors of *Pm8* and protein interaction between PM8 and

PM3 occurred in a co-immunoprecipitation assay in extracts from *Nicotiana*. A similar suppression effect was detected when two different transgenic *Pm3* alleles were combined (Stirnweis *et al.* 2014a). There, the LRR domain was identified as the causative suppression domain. This might also be true for the suppression of *Pm8* but would need to be tested further. We also observed that suppression in transgenic crosses was race-specific. First infection tests in a non-transgenic *Pm8*/*Pm3CS* cross and in *Pm8* wheat-rye translocation lines found no such effect. There, the lines carrying *Pm8* and *Pm3* were fully susceptible to all tested wheat powdery mildew isolates. Therefore, the observed race-specificity of suppression in transgenic crosses could be solely due to the overexpression of *Pm8* and *Pm3* in these lines and their differential expression caused by positional effects.

5.5 Stable deployment and use of *R* genes in breeding

In the last two decades, research in the field of plant immunity has led to a much improved understanding of how plants defend themselves against pathogens and which molecules are involved. This knowledge should now be translated into biotechnological approaches for plant breeding for a sustainable, economic, and ecological future agriculture (Eichmann and Hückelhoven 2011). The increasing number of plant genomes being sequenced will improve physical maps of genomes. Together with resequencing of accessions from different gene pools, these will boost the number of genetic markers that become available for genomic selection as well as association genetic approaches (Feuillet *et al.* 2012). The knowledge of plant immunity also leads to new strategies of improving plant resistance to pests by intervening at different stages of plant defence (Seifi *et al.* 2013). These strategies include improved pathogen recognition through pattern recognition receptors or resistance proteins, increased plant defence signalling and execution, blocked pathogen virulence genes by genetic engineering or by chemicals, and primed plants for protection against further

pathogen attack by beneficial microbes and chemicals (Gust *et al.* 2010). Transgenic approaches can be used to transfer pattern recognition receptors or resistance genes from alien sources into elite cultivars (Wulff *et al.* 2011) without transferring negative genetic material resulting in linkage drag. The identification of whole effector transcriptomes from a diverse range of pathogens in recent years has made effectors an ideal tool to identify and deploy resistance genes (effectoromics). They can be used for fast screening assays of resistance function, as well as for monitoring pathogen populations to assist chemical control and deployment of appropriate resistance genes (Vleeshouwers and Oliver 2014). New technologies such as TALENs (Schornack *et al.* 2013) and CRISPRs (Upadhyay *et al.* 2013) are becoming available in plants and allow targeted editing in genomics-assisted breeding. Our knowledge about resistance gene suppression gained in chapter 3 will also help to circumvent it in future plant breeding. This knowledge and these techniques are becoming a very important part of modern disease resistance breeding in crop plants and are contributing to the improvement of agriculture.

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